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TITLE: Research and Operational Support for the Study of Militarily Relevant Infectious Diseases of Interest to the United States Army and the Royal Thai Army

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14. ABSTRACT

Cooperative agreement # DAMD17-01-2-0005 was implemented January 1, 2001 to provide funding support for Royal Thai Army at Armed Forces Research Institute of Medical Sciences (AFRIMS) engaged in research activities in collaboration with US Army. Administrative, logistical and scientific personnel required to support the ongoing U.S. Army AFRIMS research efforts, and utilities and maintenance required to support the U.S. Army AFRIMS research effort.

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I. INTRODUCTION

A. General

Collaborative studies into infectious diseases of military importance have been conducted at the Armed Forces Research Institute of Medical Sciences (AFRIMS) by both the U.S.Army Medical Component (USAMC), and the Royal Thai Army Medical Component (RTAMC) for four decades. Studies leading to develop drugs and vaccines to combat tropical diseases of military relevant importance.

B. Statement of Work

Administrative, logistical and scientific personnel required to support the ongoing U.S. Army AFRIMS research efforts, and utilities and maintenance required to support the U.S. Army AFRIMS research effort.

C. U.S. ARMY AFRIMS Research Efforts at Department of Entomology

Department of Entomology research efforts are the following:

1. Field Evaluation of Mosquito Control Strategies in Thailand

- Evaluation of Passive Measures for the Control of Mosquitoes in a Military Setting

- Evaluation of *Anopheles* Control Methods in a Malaria-Endemic Village in Thailand

- Field Evaluation of Topical Arthropod Repellents in Thailand

2. Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines

3. Development of Chigger-Tick Surveillance Traps

4. Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

D. Department of Immunology AFRIMS FY08 Research Accomplishments

Department of Immunology research efforts are the following:

1. Human Malaria Vivax Challenge.

2. Safety and Immunogenicity of a *Plasmodium vivax* Circumsporozoite Protein in Vaccine Candidate in Rhesus macaques.

3. Preclinical Evaluation of the Safety and Immunogenicity of a Vaccine Consisting of *Plasmodium falciparum* Liver-stage Antigen 1 with Adjuvant AS01B Administered Alone or Concurrently with the RTS,S/AS01B Vaccine in Rhesus Primates.

4. Polymorphism patterns in Duffy-binding protein among Thai *Plasmodium vivax* isolates.

5. Efficacy of Artesunate-Mefloquine Combination Therapy for the Treatment of Uncomplicated Falciparum Malaria in Trat Province Thailand.

6. A Phase II, Randomized, Open-label, Dose-ranging Study of GMP Intravenous Artesunate for Optimizing Parasite Clearance in Uncomplicated *P. falciparum* Malaria.

7. Surveillance and Laboratory Characterization of Artemisinin Resistant *Plasmodium falciparum* Strains to Inform Drug Development.

8. Artemisinin Resistance in Cambodia I (ARC I).

9. Artemisinin Resistance in Cambodia II (ARC II).

10. Pharmacologic and Pharmacodynamic Animal Studies in Support of Mirincamycin Development.

11. Safety and Immunogenicity of *Plasmodium vivax* circumsporozoite Vaccine in Rhesus Monkeys.

12. Evaluation of Avian Influenza Hemagglutinnin Sequences in Wild Birds.

13. Kwai River Christian Hospital Surveillance of Influenza like illness.

14. Influenza Surveillance in Cambodia.

E. U.S. ARMY AFRIMS Research Efforts at Department of Enteric Diseases

Department of Enteric Diseases research efforts are the following:

1. Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand

2. Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens

3. Characterization of Enteric Pathogens Isolated from Children iand Adults in Maldives

4. Capsule genotyping system for *Campylobacter jejuni* and sequencing of capsule locus of *C. jejuni* type strain HS O:42.

5. Adaptation of the Established Rhesus Monkey Intragastric Challenge Model of Shigellosis to Study WRSD1 a Live Attenuated *Shigella dysenteriae-1* Vaccine Candidates

6. The production of Antisera in Non-human Primates againse Live *Shigella sonnei* 53G Strain

7. "Exempt" Human use Protocol: Establish ELISA Reference Sera to be Used for Protocol "Establishment of a *Shigella sonnei* Challenge Model for Evaluation of Future Vaccine Candidates"

8. Surveillance of Antimicrobial Resistance of enteric Pathogens in Indigenous Populations in Nepal

9. Establishment of a *Shigella sonnei* Challenge Model for Evaluation of Future Vaccine Candidates

10. Surveillance of Respiratory Pathogens in Patients Attending Royal Thai Army Hospitals

F. U.S. ARMY AFRIMS Research Efforts at Department of Veterinary Medicine

Department of Veterinary Medicine research efforts are the following:

1. Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta*)/*Plasmodium cynomolgi* Malaria Models

2. Care and Maintenance of Rhesus (*Macaca mulatta*) and *Cynomolgus (Macaca fascicularis)* monkeys and Management of Breeding Colonies

3. Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance/Quality Surveillance Program

4. A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs

5. Characterization and Validation of *Anopheles dirus* Sporozoite-Induced Mouse Malaria Models (ICR mouse/*Plasmodium berghei* and *P. yoelli*) for Screening Exoerythrocytic Antimalarial Drugs

6. Active and Passive Protection of Mice against Japanese Encaphalitis Virus

7. Institutional Animal Care and Use Committee

G. U.S. ARMY AFRIMS Research Efforts at Department of Virology

Department of Virology research efforts are the following:

1. Prospective Study of Dengue Virus Transmission and Disease in Primary Schools and Villages in Kamphaeng Phet, Thailand

2. The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue

3. A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naïve Infants

4. A Phase I/II, Open, Five-Year, Clinical Follow-Up Study of Thai Children Who Participated in Dengue-003 ("A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naïve Children") with Evaluation of a Booster Dose Given One Year after Primary Dengue Vaccination Series

5. A Phase II, Prospective, Randomized, double Blind, Placebo controlled Field Efficacy Trial of a Candidate Hepatitis E Vaccine in Nepal, WRAIR #749, HSRRB Log #A-9117.1

6. Japanese Encephalitis Surveillance in Nepal

7. Influenza Surveillance in Southeast Asia

8. Phase II, Randomized, Double-Blind, Single Center, Controlled Study of Two Doses of Different Formulations of the WRAIR Live Attenuated Tetravalent Dengue Vaccine Compared to a Placebo Control, Administered on a 0-6 Month Schedule, to Healthy Adults

9. Use of Geographic Information System (GIS) to Establish a Community-Based Biosurveillance Infrastructure in Cebu City, Philippines (WRAIR #1385)

10. Sentinel Surveillance for Emerging Diseases Causing Dengue-like or Acute Encephalitis Syndrome in the Philippines (SEDP)

11. Prospective Studies of Avian Influenza Transmission in Cambodia and Thailand

H. U.S. ARMY AFRIMS Research Efforts at Department of Retrovirology

Department of Retrovirology research efforts are the following:

1. A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp 120 B/E (AIDSVAX® B/E) Boosting in HIV-uninfected Thai Adults (RV144, HSRRB Log No. A-11048, B-IND 8795)

2. Extended Evaluation of the Virologic, Immunologic, and Clinical Course of Volunteers Who Become HIV-1 Infected during Participation in a Phase III Vaccine Trial of ALVAC-HIV and AIDSVAX® B/E (RV152, WRAIR #1184)

3. A Phase I Double-Blind Randomized Dose Escalating, Placebo-Controlled, Study of Safety and Immunogenicity of WRAIR/NIH Live Recombinant MVA-CMDR (HIV-1 CM235 env/CM240 gag/pol) Administered by Intramuscular (IM) or Intradermal (ID) Route in HIV-Uninfected Adults (RV158, WRAIR #1143)

4. Protocol G, A Cross-Sectional Study to Screen for an Generate Broadly Neutralizing Monoclonal Antibodies from HIV Infected Individuals (RV212, WRAIR #1320)

5. The Molecular Epidemiology of HIV-1 among HIV Blood Testing Clients Attending the Thai Red Cross Anonymous Clinic in Bangkok, Thailand. (RV225, WRAIR #1383)

6. HIV Specific Immune Responses in Thai Individuals with HIV Dementia (RV238, WRAIR #1418)

7. Predictors of Neuro-Cognitive Decline and Survival in HIV-Infected Subjects (SEARCH 001, WRAIR #1161)

8. Preliminary study of early primary HIV infection in high risk cohort (SEARCH 004, HSRRB # A-14273.3)

I. Space and Utilities Required

Funding under the cooperative agreement is also directed by the Principal Investigator to the provision of site maintenance including space and utilities management for both the RTAMC and the USAMC in support of research activities.

II. BODY

A. Department of Entomology, AFRIMS FY08 Research Accomplishments

1. Title of Research Project: Field evaluation of mosquito control strategies in Thailand.

Studies:

- Evaluation of Passive Measures for the Control of Mosquitoes in a Military Setting

- Evaluation of *Anopheles* Control Methods in a Malaria-Endemic Village in Thailand

- Field Evaluation of Topical Arthropod Repellents in Thailand

a. Investigators:

- Alongkot Ponlawat, Ph.D.
- MAJ Brian Evans, Ph. D.
- MAJ Jason Richardson, Ph.D
- MAJ Kendra Lawrence, Ph.D.

b. Objectives:

The goals of this set of studies are to:

1. Evaluate the efficacy of insecticide-impregnated tents on malaria vector populations. Currently, Preventive Medicine (PM) and field sanitation team personnel have responsibility for spraying tents with insecticide. If tents pre-treated with insecticide are found to be effective over the long-term, this would allow PM personnel to devote their resources to other mission essential PM issues.

2. Evaluate the efficacies of insecticide-impregnated bed nets and a barrier treatment of insecticide on malaria vector populations. The aim of this project is to evaluate potentially novel strategies of controlling malaria vectors that could be implemented by PM personnel.

3. Determine the efficacies and durations of new repellent formulations of IR3535 and picaridin relative to the Extended Duration Topical Insect and Arthropod Repellent (EDTIAR) with 33% DEET against important disease vectors such as *Aedes aegypti* and *Anopheles spp.* under field conditions in Thailand.

c. Methods:

Evaluation of Passive Measures for the Control of Mosquitoes in a Military Setting

The efficacy of ZeroFly® deltamethrin-impregnated tents (Vestergaard Frandsen, Switzerland) on controlling malaria vector populations was evaluated relative to that of non-treated tents. Twelve treated tents and twelve non-treated tents were each divided into 3 clusters of 4 tents with each cluster randomly placed into a pre-designated location in or around a village. Based on mosquito collection records from local public health officials and preliminary adult mosquito surveillance conducted by AFRIMS, a heavy breeding area of vector mosquitoes at Kang Hang Meow District in Chanthaburi province was selected as the test site. This field site is located approximately 250 km east of Bangkok

Baseline mosquito surveillance data in the absence of human subjects and tents was collected for each of the 3 months before the initiation of the treatment for each cluster location using CDC (Centers for Disease Control and Prevention) traps only (not human volunteers) to ensure that mosquito densities were relatively similar among the 6 cluster locations. A preliminary resistance assessment was conducted to determine if and at what intensity the local mosquitoes are resistant to deltamethrin. The microplate bioassay method developed by the CDC will be further utilized for the assessment of resistance.

Mosquito vector densities were determined using dry ice-baited standard miniature CDC light traps hung on the outside of and against the tents. Volunteers were screened and one volunteer randomly assigned to each tent for each of the 3 consecutive nights per month and these assignments will remain permanent for the duration of the study (18 months). Mosquitoes are collected in each tent over a 12-h period (1800 to 0600 hrs) for 3 consecutive nights at the beginning of each month.

.
Evaluation of *Anopheles* Control Methods in a Malaria Endemic Village in Thailand

Three villages (10-15 km apart) of similar size (i.e. population, number of houses, and area) were used to conduct this study. Two villages in Mae Sot District, Tak Province (Ban Khun Huai and Ban Pa Dae) were designated as treatment villages and one village as a control village (Ban Tham Sua). One treatment village (Ban Pa Dae) was provided with long-lasting insecticide-treated bed nets (LLIN) containing deltamethrin (PermaNet™). The second treatment village received a one-time barrier treatment of bifenthrin (TalStar One™) applied to a 20 m swadth of vegetation surrounding the perimeter of the village. The control village received no treatment. The number and sizes of LLINs and non-treated bed nets were dependent on the sleeping arrangement of each family. Coordination was made with the village chief and district pesticide applicators prior to the application of a barrier treatment. Mosquitoes were collected over a 12-h period (1800 to 0600 hrs) for 3 consecutive nights per month per village for each of the 3 months prior to the LLIN/barrier treatments and monthly for the 18 months immediately following the treatment using the method described above.

Fifteen houses from each village were selected by simple random sampling for each of the 3 collection nights as locations where collections were to be performed. A sample size of 45 homes (15 homes per night for 3 nights) in each village has 80% power to detect a difference in means of 24 mosquitoes per trap/night assuming that the common standard deviation is 40 using a two-sided t-test with a 0.050 level of significance. One healthy volunteer (20 to 50 years of age) from each collection location (home) was assigned to sleep under the bed net. Mosquito vector densities were determined using dry-ice baited standard miniature CDC (Centers for Disease Control and Prevention) light traps with the inlet of each trap positioned 25 cm. above the foot of a volunteer sleeping/lying on the floor of a bed net.

Field Evaluation of Topical Arthropod Repellents in Thailand

Volunteers in each treatment group will have one leg treated with the assigned topical repellent while the other leg remains untreated. A 600 cm^2 treatment area from just above the ankle to just below the knee will be determined for each volunteer. Five (5) equally spaced circumference measurements will be taken along each lower leg, averaged and divided into 600 to get the length of the exposed area. This area will then be defined above and below by an indelible marker line on both legs. Each volunteer will then wash their legs below the knees with a mild soap and water. The marked area from ankle to knee on one leg will be treated with the assigned topical repellent and the same marked area on the opposite leg will be left untreated to serve as a control. The Environmental Protection Agency (EPA) Product Performance Test Guidelines recommend using between 1.0 and 1.5g of cream or lotion and 1.0g of pump spray or liquid over 600cm^2 of skin surface area for testing repellents. Using these guidelines, 1.5g of the lotions and creams and 1.0g of the sprays will be spread evenly on the treatment area using a talc-free glove. During the mosquito challenge, volunteers will be covered with mesh jackets, long sleeve shirts, long pants, and footwear except for the exposed experimental areas. All mosquitoes landing in the marked areas of the exposed lower legs will be counted and mouth-aspirated during a 50-minute test period and placed into screen-topped cartons (either treatment or control) and marked with collection data (date, time of collection, collector number, etc.). Studies were conducted in two areas of Thailand – Lop Buri and Chantaburi.

Lop Buri:

There were 8 days of participation: one day for screening and training, at least 6 days of testing, and one day for a post-study follow-up 72 hours after study completion. On the first day, volunteers were screened and assessed for study eligibility by the Principal Investigator (PI) or Associate Investigators (AI). After providing informed consent, all female volunteers provided a urine specimen to determine pregnancy prior to any participation in the study. Pregnancy tests were read and female volunteers knew their results prior to study participation.

Also on the first day, volunteers were randomly assigned to one of five treatments (repellent formulation) and remained in that group for the remainder of the study. Each

volunteer was challenged at 6 post-application time points (2, 4, 6, 8, 10 and 12 hours). Peak biting activity of *Aedes aegypti* occurs twice daily (0800-0900 and 1600-1700 hours) and the afternoon peak biting period was selected for repellent evaluation due to the extended period of time required between repellent application and evaluation. A volunteer can only be challenged at one post-application time point each day (2, 4, 6, 8, 10, or 12 hours) such that at least six days are required to capture all 6 post-application time points. Thus, a staggered application design was employed so that all post-application challenge time points were measured during the peak biting time. Peak biting activity can vary depending on time of year, weather conditions, and village location. For each repellent trial, the peak biting activity was verified prior to study execution using the most recent Human Landing Count (HLC) data and/or surveillance data collected several weeks prior to initiation of the trial. The goal was to detect a difference in efficacy for duration periods of 2, 4, 6, 8, 10, and 12 hours between the treated legs and the negative control legs.

Chantaburi:

There were 5 days of participation: one day for screening and training, at least 3 days for testing, and one day for a post-study follow-up 72 hours after study completion. On the first day, volunteers were screened and assessed for study eligibility by the PI or AI. All female volunteers provided a urine specimen for the pregnancynt test after providing informed consent. Also on the first day, volunteers were randomly assigned to one of five treatments (repellent formulation) and remained in that group for the remainder of the study. Each volunteer was challenged at 6 post-application time points (2, 4, 6, 8, 10 and 12 hours). Peak biting activity of anopheline mosquitoes (*Anopheles annularis* and *An. barbirostris* group) occurs for approximately 3 hours per night (between 2000 and 2300 hours); therefore, a volunteer can only be challenged at two post-application time points each night (2 and 4 hours; 6 and 8 hours; or 10 and 12 hours) such that at least three days are required to capture all 6 post-application time points. Thus, a staggered application design was employed so that all post-application challenge time points are measured during the peak biting time. However, peak biting activity can vary depending on time of year, weather conditions, and village location. Prior to the execution of each repellent trial, the peak biting activity was verified using the most recent Human Landing Count (HLC) data and/or surveillance data collected a couple weeks prior to initiation of the trial. The goal was to detect a difference in efficacy for duration periods of 2, 4, 6, 8, 10, and 12 hours between the treated legs and negative control legs.

d. Results:

1. Zerofly® tents and non-treated tents were set up in the field at Kang Hang Meow district, Chantaburi province. Twenty four volunteers were recruited into the study in October 2008 after the human use protocol was approved by theThai MoPH. Mosquitoes have been collected using the CDC light traps over a 12-h period (1800 to 0600 hrs) for 3 consecutive nights per month since October 2008. The densities/species of mosquitoes collected from the CDC light traps is continuing to be monitored monthly.

2. An evaluation of *Anopheles* control methods (barrier treatment and LLINs) began in January 2009 after the human use protocol was approved by the Thai MoPH. One treatment village (Ban Pa Dae) was provided with LLINs (PermaNet™). The second treatment village (Ban Khun Huay) received a one-time barrier treatment of bifenthrin (TalStar One™) applied to vegetation around the perimeter of the village. The control village (Ban Tham Sua) received no treatment. Mosquitoes have been collected using the CDC light traps over a 12-h period (1800 to 0600 hrs) for 3 consecutive nights per month. The densities/species of mosquitoes collected from the CDC light traps is continuing to be monitored monthly.

3. The human use protocol for the repellent project received approval from the WRAIR IRB and the Thai MoPH in July 2008. The efficacies of repellents against *Ae. aegypti* were determined in Lopburi province in August 2008. The efficacies of repellents against malaria vectors (*Anopheles* spp.) were evaluated in Kang Hang Meow district, Chantaburi province in November 2008. Data from the human repellent studies is currently undergoing statistical analysis for subsequent publication and will be included in the 2009 CA report.

e. **Future Plans:** Both the tent and barrier treatment/bed net studies described above are due to be completed within the next 12 to 14 months. Follow-on studies will evaluate the efficacies of spatial repellent devices and an insect growth regulator on vector densities.

2. Title of research project: Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines

a. **Investigators:**

- MAJ Jason H. Richardson, Ph.D.
- Dr. Kriangkrai Lerdthusenee, Ph.D.

b. **Objectives/Methods:**

1. Conduct genetic characterization of the 12 strains of *Orientia tsutsugamushi* infecting AFRIMS colonies of *Leptotrombidium* mite spp. in order to determine phenotypic and genetic relationships between strains.

2. Assess the ability to transmit *O. tsutsugamushi* to laboratory mice for each of the 12 mite colonies using the animal passage procedure. Detection of the presence of *O. tsutsugamushi* in animal tissues and chigger specimens will be examined by conventional PCR techniques.

3. Focus efforts on building up chigger colonies sufficient to support vaccine challenge studies. Select the highest infection rate from our 12 scrub typhus infected chigger-mite colonies and mass produce its population to support the vaccine challenge studies. The rearing and maintenance of *Leptotrombidium* chiggers is a long and slow

process. The complete life cycle of *Leptotrombidium* mites (from egg to egg-laying adult) requires approximately 3 months. At a minimum, 6 months is required to build up the colony to a level sufficient to support studies in our laboratory.

4. Develop methods and conduct studies to evaluate the efficacies of candidate scrub typhus vaccines using the chigger-mouse model. Determine the course of rickettsemia over time in the Lc-1 strain of *O. tsutsugamushi* and develop and/or confirm diagnostic procedures (PCR, ELISA, etc.) to quantify rickettsemia in challenged mice.

c. Results:

1. Evaluations of scrub typhus vaccine candidates Kpr56 & pKarp 47 revealed similar efficacies on vaccinated mice when challenged either by needle injection of homogenized scrub typhus-infected (Lc-1) chiggers or by the natural chigger-feeding method. Vaccinated mice exhibited prolonged death when compared with the reference group. Although the Kpr56-vaccinated mice exhibited slightly longer survival than the pKarp 47-vaccinated mice, statistical analysis exhibited no significant differences between survival times.

2. Genomic DNA from the 56 kDa gene of *O. tsutsugamushi*-infected *L. chiangraiensis*-1 (Lc-1) chigger was extracted and then sequenced. Based upon the 56 kDa type-specific antigen (tsa) gene, the nucleotide sequence of *O.tsutsugamushi* extracted from scrub typhus-infected *L. chiangraiensis*-1 (Lc-1) was described with a total of 1,590 basepairs. The *O. tsutsugamushi* Lc-1 sequence was 96% identical to *O. tsutsugamushi* strain UT169 and *O. tsutsugamushi* strain TW45R was 93% identical to the *O. tsutsugamushi* Karp-strain. The complete nucleotide sequence was submitted and deposited in GENBANK under the title of "Genotypic identification of *Orientia tsutsugamushi* isolated from *Leptotrombidium chiangraiensis* mite by sequencing of 56 kDa gene" (bankit1137375 FJ374771).

d. Future Plans:

1. Establish the optimum inoculation doses {the lethal dose 50% (LD_{50}) and infectious dose 50% (ID_{50})} of homogenized *O. tsutsugamushi*-infected mice tissue samples (spleen/liver).

2. Evaluate the natural challenge on Lc-1 homologous vaccinated mice.

3. Title of research project: Development of Chigger-Tick Surveillance Traps

a. Investigators:

- MAJ Jason Richardson, Ph.D.
- Dr. Kriangkrai Lerdthusenee, Ph.D.

b. Objective: Develop surveillance techniques and/or tools for rapid quantitative assessments of ectoparasites.

c. Methods:

Chigger Trap

Black plates (set of black squares, size 5" x 5") were previously used by PM personnel to evaluate chigger densities. The top surface of the square sometimes contained an adhesive substance (double-sided tape or tanglefoot) capable of trapping chiggers. Several other designs had been developed by AFRIMS in conjunction with the black-plate model in order to enhance performance (i.e., the cotton-tube, cotton-plate and burrow type traps). In addition, the use of an arthropod attractant such as a portable source of CO_2 or an Insectigator Sachet on the surface of black plates could enhance the effectiveness of a trap.

Determination of a Chigger's Normal Walking Distance

Our objective was to establish the mean walking distance of a chigger population per unit of time and then compare this result to the mean walking distance in the presence of CO_2. In order to better understand chigger host-seeking behavior, we developed a behavioral assay to measure the distance chiggers will move with and without the addition of CO_2. In the first set of experiments, we built a 50-cm track suspended within a Plexiglas chamber. The track was made of a mixture of charcoal and Plaster of Paris and was designed with a small groove (0.5 cm in depth) to serve as a channel through which the chigger could walk. For each of 40 replicates, 10 chiggers were released at one end of the charcoal track. After 60 minutes, each chigger was scored with regard to the distance it had traveled down the track. Of the 400 chiggers observed, a total of 352 chiggers were retrieved, while 48 chiggers were lost during the tests. Approximately two-thirds of the chiggers remained within 10 cm of the release point. The remaining one-third of chiggers was found within a range of 10 to 45 cm from the release point. None of the chiggers walked passed the 45 cm mark.

Evaluation of CO_2 as an attractant to chiggers

In order to determine the efficacy of an attractant to our chigger traps, CO_2 was released into the chamber at the end opposite to the chigger release point. CO_2 was released from a CO_2 tank at a controlled rate of approximately 7.5 ml/min. This rate attracted the highest number of chiggers. Based on the results above, a walking distance of no more than 10 cm was considered normal behavior. Any distance beyond 10 cm was assumed to be the result of an attractant. Forty replicates (10 chiggers/replicate) were conducted for a total of 400 chiggers. Of this number, 358 chiggers were retrieved while 42 chiggers were lost during the tests.

Evaluation of a "Prototype Chigger Trap Model" with CO_2 as Attractant

CO_2 was applied on a prototype chigger trap (black cloth pyramid on black plate). Four different traps were compared in this experiment as follows: black plate (control), prototype chigger trap without CO_2, prototype chigger trap with CO_2 (from CO_2-tank) and a prototype chigger trap with CO_2 (from a CO_2 sachet). Either 2 or 4 chigger traps were placed 10 cm from the center of a charcoal box platform. Forty chiggers were released from the center of the platform. Chiggers were allowed to move/walk naturally for a 60-min duration. After this period of time, any chiggers remaining on the traps were counted.

d. Results:

1. With the addition of CO_2 at one end of the chamber, approximately 30% of tested chiggers moved/walked within the "normal walking zone (0-10 cm), while almost 70% of tested chiggers were found approaching the attractant zones (> 10 cm). Significant differences ($p < 0.05$) were found between the mean numbers of chiggers appearing in the two zones. Therefore, we assumed that tested chiggers were attracted by the presence of CO_2.

2. In the prototype chigger trap model laboratory study, the mean numbers and percentages of chiggers appearing on the traps were as follows: 0.70 (1.75%), 5.50 (13.75%), 12.00 (30.00%) and 11.50 (28.75%) for black plates (control), prototype chigger traps without CO_2, prototype chigger traps with CO_2 from tank and prototype chigger traps with a CO_2 sachet, respectively. Significant differences ($p < 0.05$) were found in the mean number of chiggers appearing in the prototype chigger trap without CO_2 and the prototype chigger trap with CO_2 (from tank) or the prototype chigger trap with CO_2 (from sachet) (One-way ANOVA/Tukey HSD). There were no significant differences ($p < 0.05$) between the prototype chigger trap with CO_2 (from tank) and the prototype chigger trap CO_2 (from sachet). The prototype trap augmented with a CO_2 source is potentially viable tool for collecting chiggers in the field

e. Future Plans: Additional prototypes and the evaluation of additional trap modifications will be necessary before coming to a consensus on the most effective surveillance tool. Modification of the evaluation model may also be necessary.

4. Title of Research Project: Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

a. Investigator:

Dr. Jetsumon Prachumsri, Ph.D

b. Objectives:

1. Provide mosquitoes with consistent, reproducible salivary gland infections to support *Plasmodium vivax* sporozoite challenge studies.

2. Provide live *P. vivax* sporozoite-infected mosquitoes and/or harvested, purified *P. vivax* sporozoites (on wet- or dry-ice) to WRAIR/NMRI investigators or collaborating institutions.

3. Provide slides of blood and exo-erythrocytic stages of *P. vivax* parasites for vaccine studies.

4. Provide qualified *P. vivax*-infected mosquitoes that can be used for human challenge studies.

c. Methods:

1. Continue to collect blood samples from patients after blood smears are confirmed by Ministry of Public Health (MOPH) microscopists as malaria-positive samples. As part of an approved Human Use Protocol, Department of Entomology personnel are allowed to draw 20 ml of a patient's blood to feed mosquitoes using a membrane feeding technique. Studies are conducted weekly at Mae Kasa and Mae Sot malaria clinics. Aliquots of each blood sample are spotted on filter paper and smeared on glass slides. Confirmation of parasite species is accomplished by PCR of filtered blood and by microscopic examination of blood smears. Infected mosquitoes are returned to AFRIMS and maintained in the AFRIMS insectary. Five to 10% of mosquitoes from each mosquito feed are checked for the presence or absence of oocysts approximately 7-10 days after infection. These mosquitoes are thereafter available for use in malaria sporozoite challenge studies.

2. Refined Sporozoite Challenge System. In 2008 we prepared mosquitoes to be hand-carried to WRAIR as a first trial. The goal was to develop a system that will i) consistently provide mosquito infection rates with >90% of blood-fed mosquitoes having +3/4 (>100 sporozoites) salivary gland infections, and ii) provide *P. vivax*-infected mosquitoes that do not harbor concomitant pathogens. Consistency in the challenge is a critical component of any vaccine trial.

3. Parasite Characterization: This is part of routine quality assurance to obtain and use parasites with similar genetic compositions so results can be compared. In the absence of an in vitro culture system, it is necessary to feed mosquitoes on a *P. vivax*-infected volunteer or on blood from a volunteer. Since it is impossible to ensure that mosquitoes are infected with a single *P. vivax* clone (as is currently done with *P. falciparum*), it is critical that a method be in place to characterize the parasites (i.e., genetic diversity of the parasites, resistance to antimalarial drugs, etc.). Once mosquitoes are infected, parasites from the infectious blood meal are characterized by PCR using polymorphic gene targets, such as the nonapeptide repeat region of the

circumsporozoite protein (PvCSP), and the region between interspecies conserved blocks 5 and 6 of the merozoite surface protein (PvMSP1).

4. There was an effort to develop a freezing system that can maintain viability and infectivity of *P. vivax* gametocytes. Varieties of cryoprotectant and freezing conditions were evaluated.

d. Results

1. An updated standard operating procedure (SOP) has been prepared for the 2008-9 studies.

2. Sporozoites have been produced and used for preparation of IFA slide antigens and for the *in vitro* culture of liver stage parasites. An *in vitro* culture (HC04) of *P. vivax* parasites in human liver cells has been studied and validated for screening of antimalarial compounds. Parasite-infected liver cells were collected and prepared for the study of parasite genes and proteins.

3. Most of the cryoprotectants and freezing conditions evaluated maintained viability of asexual stage parasites with the exception of the gametocytic stages.

4. The transportation of *P. vivax*-infected mosquitoes to WRAIR proved to be successful. More than 80% of the mosquitoes survived during transport and up through 2 weeks post-feeding. Sprorozoite rates were found to be high and this method could be used for further studies at WRAIR.

e. Future Plans:

1. Continue to provide *P. vivax* parasites to support several drug and vaccine development studies. Blood collection from patients will continue in order to provide parasites for further studies.

2. We anticipate to collect 250-300 blood samples in 2009 and will provided 2-3 batches of infected mosquitoes for a human challenge study at WRAIR.

B. Department of Immunology AFRIMS FY08 Research Accomplishments

1. Title of Research Projects:

1. Human Malaria Vivax Challenge. Status: Protocol in development undergoing ethical review.

2. Safety and Immunogenicity of a *Plasmodium vivax* Circumsporozoite Protein Vaccine Candidate in Rhesus Macaques. Status: Study in life completed, laboratory analyses are ongoing.

3. Preclinical Evaluation of the Safety and Immunogenicity of a Vaccine Consisting of *Plasmodium falciparum* Liver-Stage Antigen 1 with Adjuvant AS01B Administered Alone or Concurrently with the RTS,S/AS01B Vaccine in Rhesus Primates, Status: Study in life completed.

4. Polymorphism Patterns in Duffy-binding Protein among Thai *Plasmodium vivax* Isolates. Study completed, and results published (PMID 18582360).

5. Efficacy of Artesunate-Mefloquine Combination Therapy for the Treatment of Uncomplicated Falciparum Malaria in Trat Province Thailand. Status: Study ongoing in liaison with Thai MoPH

6. A Phase II, Randomized, Open-label, Dose-ranging Study of GMP Intravenous Artesunate for Optimizing Parasite Clearance in Uncomplicated *P. falciparum* Malaria. Status: Clinical trial completed.

7. Surveillance and Laboratory Characterization of Artemisinin Resistant *Plasmodium falciparum* Strains to Inform Drug Development. Status: Validation of standard clones near completion.

8. Artemisinin Resistance in Cambodia I (ARC I). Status: Manuscript published.

9. Artemisinin Resistance in Cambodia II (ARC II). Status: Clinical Trial ongoing.

10. Pharmacologic and Pharmacodynamic Animal Studies in Support of Mirincamycin Development. Status: Bio-availability and tolerability study in primates completed. Efficacy testing to be initiated.

11. Safety and Immunogenicity of *Plasmodium vivax* circumsporozoite Vaccine in Rhesus Monkeys. Status: Completion of in-life phase; laboratory immunology assays are ongoing.

12. Evaluation of Avian Influenza Hemagglutinnin Sequences in Wild Birds. Status: Data generation in progress. In life complete; publication submitted.

13. Kwai River Christian Hospital Surveillance of Influenza like illness. Status: Surveillance on-going.

14. Influenza Surveillance in Cambodia. Status: Assessment trip made and protocol drafted.

a. Investigators:

- Dr. Mark Fukuda, MD
- Dr. Bryan L. Smith, MD
- Dr. Sathit Pichyangkul, Ph.D.

- Dr. Paktiya Teja-Isavadharm Ph.D.
- Dr. Krisada Jongsakul, MD
- Dr. Wiriya Rutvisutinunt
- Dr. Delia Bethell, MD
- Dr. Youry Se, MD
- Dr. Kurt Schaecher, PhD
- Dr. Daniel L. Saunders
- Dr. Stuart D. Tyner
- Dr. Ans Timmermans
- Dr. Jessica Lin
- Dr. Lon Chanthap

b. Objectives:

1. To protect, project and sustain the military soldier against disease threats produced by the two major species of malaria, *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv).   To support this mission through the evaluation of new or improved vaccines, prophylactic and therapeutic drugs, rapid diagnostic kits, and the maintenance of a center for excellence focused on the basic biology and epidemiology of malaria.

2. To assess emerging febrile diseases along high-risk regions in Thailand and throughout SE Asia.

c. Methods:

The Department of Immunology and Medicine has applied as many kinds of classical and state-of-the-art technologies as possible to the above multi-faceted research.   Clinical research included mobile epidemiology team able to work in adverse conditions where malaria is present, including field sample collection and processing screening, reference microscopy, assessment of rapid diagnostics for various tropical infectious diseases, and a staff well-versed in conduct of clinical trails to GCP and ICH standards.   The animal research teams are all trained in laboratory animal research and regulations, current AALAAC requirements, and laboratory animal test and observation methods.   State-of-the art methodologies are available for the study of vaccine and drugs to include advanced molecular biology methods such as sequencing, SNP analysis, and real-time PCR. Cellular immunology techniques are available which include flow cytometry and sorting technologies, ELISPOT, and molecular methods. Pharmacology assays include HPLC, LC-MS, a unique malaria bioassay to measure the *in vivo* antimalarial bioactivity of potential new antimalarial medications, sustained malaria cell culture and radioisotopic uptake, and antibody based methods for measuring *in vitro* drug sensitivity patterns of malaria strains against standard malaria drugs.

d. Results (accomplishments during the period of January-December 2008):

1. Malaria Drug Development

Managed the implementation of departmental quality practices for the execution of studies in agreement with MRMC policies and US FDA standards in support of IV AS drug development program. Work involved the generation and/or revision of nearly 60 SOPs; upkeep of personnel training and qualification records; space utilization for LCMS lab, sample repository, and field clinical lab; establishment of a controlled sample tracking and inventory system; qualification of equipment used for regulated studies; and continued interaction with Medical Maintenance and service contractors. Helped integrate Departmental QA/QC efforts with those of the subsequently established QA units at the AFRIMS, WRAIR and MRMC. Participated in the IPT teleconferences, providing metabolism and pharmacokinetics insight.

Parenteral antimalarial drugs are indicated for the treatment severe malaria and when oral therapy cannot be given. The goals of treatment are prevention of death and reduction of morbidity. Rapid reduction in parasite clearance appears to be a good surrogate for these endpoints. While artemisinins is known to clear malaria more rapidly than other antimalarials, the available data on optimal dosing for pharmacodynamic effect is difficult to interpret and does not include the higher or multiple daily doses that are now in common clinical usage. As part of the US Army's goal to secure FDA approval of a GMP formulation of intravenous artesunate, we conducted a multi-center, phase II, open-label, dose-ranging study in 33 Thai adults and 67 Kenyan children and adults with uncomplicated *P. falciparum* malaria. Safety, tolerability and efficacy data were collected and pharmacodynamic endpoints including parasite reduction ratios and parasite and fever clearance were determined, and compared between treatment arms.

The Pharmacology Section completed work in support of the WRAIR DIV ET product development portfolio in FY 2008. Imidazoladinediones have been shown to have useful properties against dormant liver stage malaria parasites in non-human primates previously. However, apparent oral bioavailability and efficacy were poor. The pharmacokinetics and pharmacodynamics of novel imdazoladinedione development candidates in non-human primates was studied. Oral bioavailability of the novel candidates was determined to be good, despite suboptimal efficacy. This prompted the synthesis of new candidates to develop a more complete structure-activity relationship within the class. A poster on the work was presented at the American Society of Clinical Pharmacology and Therapeutics.

Mirincamycin is a lincosamide antibiotic structurally related to clindamycin. Prior work in the 70s and 80s led to discovery of antimalarial properties in primates, but the drug was never tested in humans. Recently, interest in this compound has resurfaced. A formal oral bioavailability study of this drug was conducted in non-human primates, and was found to be roughly 10-13% compared to intravenous administration. The drug was reasonably well tolerated, and formal efficacy testing will be conducted shortly.

2. Malaria Drug Resistance Surveillance

Artemisinin based combination therapies (ACTs) are the first line treatment for drug resistant *Plasmodium falciparum* malaria. The current major global investment in ACTs is threatened by the possible emergence of resistance to artemisinins, as signaled by a trend of increasing ACT treatment failure on the Thai-Cambodian border, which has historically been an epicentre of drug resistant malaria. Artesunate in combination with mefloquine has been the first-line drug for uncomplicated falciparum malaria on the Thai side of the border since 1995 and in Cambodia since 2000. Therapeutic efficacy monitoring is regularly conducted by both the Thai and Cambodian malaria control programs. Both progressively increased parasite clearance times and unusually high failure rates with artesunate-mefloquine have been reported recently on both sides of the border.

AFRIMS began working in collaboration with the Thai MOPH in Trat Province, Thailand to try and determine why the treatment failures described by the Thai National Malaria Program (Vijaykadja, 2006) were occurring. An integrated *in vivo-in vitro* approach was adopted using existing protocols. This approach comprised antimalarial treatment in accordance with MOPH guidelines (directly observed treatment with AS (6mg/kg daily for 2 days), MQ (25mg/kg split into 2 doses) and PQ (0.5mg/kg single dose on Day 2) with all doses given as DOT), and *in vitro* culture of parasites with drug sensitivity assays at admission to the study and subsequently if treatment failure occurred. Parasite growth inhibition was used as a measure for drug sensitivity of fresh samples in a HRP2 double-site antigen capture ELISA. Follow-up had previously been to Day 28 in accordance with WHO guidelines (WHO 2003) but was extended to 42 days when AFRIMS became involved since this is the preferred duration of follow-up following MQ therapy. We found that the PCR-corrected ACPR (cure rate) at 42 days for Trat in 2005 was 81% (7 out of 42 enrolled patients failed therapy and 5 were reinfected). The second (and currently on-going) Trat study, (WRAIR #1327) started in September 2007 and has enrolled 13 patients to date. The study also uses an in vivo/in vitro approach yet incorporates a more detailed human use (*in vivo*) study, with plasma drug level measurements and a comparison of 2 and 3 days AS treatment. AFRIMS and the Thai

MOPH will continue to work in collaboration during the forthcoming study. The *in vivo* component aims to compare the efficacy and tolerability of artesunate (12mg/kg) and mefloquine (25mg/kg) given over 2 or 3 days for the treatment of uncomplicated *P. falciparum* malaria in Trat Province, Thailand. This has important public health implications as it may influence future treatment policy.

In our recent ARC1 study ("Artemisinin Resistance in Cambodia 1", WRAIR #1296, HSRRB A-13922) conducted in 2006/7 in Ta Sanh, Western-Cambodia, individual isolates were detected that are highly suggestive of resistance to artemisinins. ARC1, which compared an experimental monotherapy regimen of 4 mg/Kg of oral artesunate for 7 days (28 mg/Kg total dose) versus a standard comparator regimen of oral quinine and tetracycline, found 4 patients in the artesunate arm who had re-emergence of *P. falciparum* parasites during 28 days of follow-up; 3 were classified as LPF and 1 as a

LCF. Mean PCT in the 4 patients who failed artesunate monotherapy was almost twice that of those who were cured (97.6 vs. 52.2 hrs). In vitro drug susceptibility tests indicate significantly higher geometric mean IC50s for artemisinins as compared to western Thailand and Bangladesh. Patients who failed therapy had IC50 values up to 5 times higher than the overall mean. Drug levels measured 90 and 150 minutes after drug intake on day 0 were used to define individual DHA levels. Of the 4 patients who failed therapy, two had satisfactory drug levels while the other two did not. Although some failures may therefore be linked to inadequate drug levels, at least 2 patients (3.3%; 95% CI: 0.4-11.5) with the highest artemisinin IC50s and PCTs of 133 and 95 hrs failed therapy in spite of adequate drug levels suggesting clinically significant resistance to artemisinins.

The currently on-going ARC2 protocol is a follow-up study to ARC1 in which 150 volunteers with acute uncomplicated falciparum malaria will be randomly assigned one of 3 arms to be treated with artesunate monotherapy (under DOT) for 7 days with doses of either 2, 4 or 6 mg/kg/day at a ratio of 2:1:2. The aim of this project is to determine whether regimens with increased artesunate doses can overcome the problem of reduced drug sensitivity to artemisinins and to determine whether these experimental regimens are safe and well tolerated.

With regard to characterization of molecular aspects of malaria infections associated with resistance to drug therapy along the Thai-Cambodian border, one study was conducted to test validity of a candidate molecular marker as a predictor for antimalarial drug resistance against *Plasmodium falciparum* malaria. Sequence analysis of *Plasmodium falciparum* SERCA-type PfATPase6 (a key target of artemisinin derivatives) in Pf Field Isolates collected from Southeast Asia suggest that in SE Asian strains there is no correlation between clinical outcome and SERCA-type PfATPase6 sequences.

In vitro drug sensitivity assays have been used as a tool to characterize the drug susceptibility phenotype of clinical *Plasmodium falciparum* isolates and to screen new candidate drugs in development. Variability in *in vitro* drug sensitivity testing throughout the malaria research world makes comparison between different data sets, different labs, and different time periods difficult. In order to develop a testable model system for generating IC50 values with patients' specimens, we need to evaluate standard clone dynamics as a mechanism to establish a validated control. In 2008, the in vitro team at the AFRIMS Department of Immunology and Medicine studied 1: the effect of long term culture of W2, D6 and 3D7 clones on measured IC50 values, and 2: the effect of time interval and frequency of sorbitol treatment on synchronization of W2 cultures.

After completion of validation of W2 standard clones, we plan to conduct drug sensitivity assays to test artemisinin-like new chemical entities (NCEs) on blood samples containing malaria parasites, both from patients that have successfully completed and that have failed artemisinin treatment. Funding for anti-malarial drug resistance is sourced from DoD-GEIS , WHO, MMV and the Bill and Melinda Gates Foundation.

3. Vaccinology and Immunology Studies in Support of Malaria Vaccine Program and Highly Pathogenic Avian Influenza Pathogenesis Studies

Several lines of evidence suggest that targeting pre-erythrocytic-stage parasites for *Plasmodium falciparum* malaria vaccine development can provide sterile immunity. We conducted a preclinical evaluation of the safety and immunogenicity of a vaccine consisting of *Plasmodium falciparum* liver-stage antigen 1 with adjuvant AS01B administered alone or concurrently with the RTS,S/AS01B vaccine in rhesus primates.

Using a rhesus monkey model, we found that LSA1 formulated with the GlaxoSmithKline proprietary adjuvant system AS01B (LSA1/AS01B) was safe and immunogenic, inducing high titers of antigen-specific antibody and CD4+ T-cell responses, as monitored by the production of interleukin-2 and gamma interferon, using intracellular cytokine staining. RTS,S/AS01B vaccination was well tolerated and demonstrated robust antibody and moderate CD4+ T-cell responses to circumsporozoite protein (CSP) and HBsAg. Positive CD8+ T-cell responses to HBsAg were detected, whereas the responses to CSP and LSA1 were negligible. For both LSA1/AS01B and RTS,S/AS01B, no statistically significant differences were observed between individual and concurrent administration in the magnitude or duration of antibody and T-cell responses. Our results revealed that both pre-erythrocytic-stage antigens were safe and immunogenic, administered either separately or simultaneously to rhesus monkeys, and that no significant immune cross interference occurred with concurrent separate-site administration.

Since the re-emergence of *Plasmodium vivax* in and around the demilitarized zone (DMZ) of South Korea in 1994, 10,000 to 20,000 new cases of *P. vivax* malaria have been reported. As high as 10% of the native population in the DMZ has sero-converted against *P. vivax* malaria. *P. vivax* malaria has also been shown to be a potential threat in Afghanistan, Iran, and Iraq, three other areas strategically important to the U.S. military. Currently, no vaccine exists against *Plasmodium vivax*, a potential threat to military operations.

Our department tested safety and immunogenicity of a *Plasmodium vivax* Circumsporozoite Protein (CSP) Vaccine Candidate in Rhesus macaques. Our aim was to measure the monkey antibody and cell mediated immune responses to a chimeric full length CSP construct (VK210/VK247 type) vaccination. We hypothesized that immunization with our construct will produce a significant increase in antibodies capable of inhibiting sporozoite invasion and cellular based immune responses relative to the pre-immune baseline. As a secondary hypothesis, we expected monkeys receiving AS01B would have higher responses relative to AS02A recipients. Laboratory analyses are ongoing.

The Duffy-binding protein II of *Plasmodium vivax* (*PvDBPII*) has been considered as an attractive target for vaccine-mediated immunity despite a possible highly polymorphic nature. Among seven *PvDBP* domains, domain II has been shown to exhibit a high rate of non-synonymous polymorphism, which has been suggested to be a

potential immune (antibody binding) evasion mechanism. We studied the extent of genetic polymorphisms and positive natural selection at domain II of the *PvDBP* gene among a sampling of 30 Thai *P. vivax* isolates. After PCR amplification of the *PvDBPII* gene and characterization of the patterns of polymorphisms in these isolates using DNA cloning and sequencing, we found a higher rate of non-synonymous and synonymous mutations suggesting that *PvDBPII* antigen appears to be under selective pressure. Polymorphisms within *PvDBPII* indicated that Thai vivax malaria parasites are genetically diverse. Phylogenetic analysis of DNA sequences using the Neighbour-Joining method demonstrated that Thai isolates shared distinct alleles with *P. vivax* isolates from different geographical areas. Our study will be valuable for the development of *PvDBPII*-based malaria vaccine.

Other immunology studies have focused on the immunopathogenesis of avian influenza using Thai viral isolates. We have found high susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-alpha and TLR ligands. There is worldwide concern that the avian influenza H5N1 virus, with a mortality rate of >50%, might cause the next influenza pandemic. Unlike most other influenza infections, H5N1 infection causes a systemic disease. The underlying mechanisms for this effect are still unclear. In this study, we investigate the interplay between avian influenza H5N1 and human dendritic cells (DC). We showed that H5N1 virus can infect and replicate in monocyte-derived and blood myeloid DC, leading to cell death. These results suggest that H5N1 escapes viral-specific immunity, and could disseminate via DC. In contrast, blood pDC were resistant to infection and produced high amounts of IFN-alpha. Addition of this cytokine to monocyte-derived DC or pretreatment with TLR ligands protected against infection and the cytopathic effects of H5N1 virus.

4. Influenza Surveillance (GEIS)

This GEIS-funded project will allow for ongoing surveillance of Influenza-Like-Illnesses (ILIs) and detection of influenza and highly pathogenic influenza among vulnerable groups who are not included in other surveillance mechanisms. Currently, we conduct ILI surveillance along the Thai-Burma border and we plan to start surveillance in Western-Cambodia early 2009.
Our sites will report the number of ILIs seen, rapid diagnostics performed, rapid diagnostics positive, and samples sent for confirmatory testing. Surveillance objectives include collecting and characterizing influenza and other respiratory viruses circulating within the human population in Thailand, Burma and Cambodia; providing data for discussion towards the annual re-formulation of the influenza vaccine; and evaluating novel molecular platforms to identify respiratory pathogens will be achieved.

There is no mechanism by which the US DoD or the international community can obtain direct information on the presence or activity of influenza or epidemics within the country of Burma. Nor does it seem that this situation is likely to improve in the near future. The closest we can come to obtaining direct information is through surveillance of Burmese migrants and/or refugees. The Kwai River Christian Hospital in

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Kanchanaburi Province is situated next to Burma and provides care to not only the residents of Kanchanaburi, but also a significant number of people who cross over from Burma seeking better medical care. Our department operates a research station in KRCH hospital.

Since the beginning of the study in May 2007 to date, a total of 300 specimens were collected. Rapid antigen testing of 298 specimens revealed 22 (7.4%) cases of Influenza A and 60 (20.13%) cases of Influenza B. Real-time PCR testing confirmed 137(45.8 %) total positive samples out of 299 specimens testes: 20 samples (6.7%) were Flu A/H1N1, 20 samples (6.7%) were Flu A/H3N2, and 97 (32.4%) were Flu B.

To contribute to the expansion of the DoD GEIS current surveillance efforts in critical geographic regions, and supported by the Cambodian CDC, we plan to initiate ILI-surveillance in Battambang, Western-Cambodia.

As in other Asian influenza surveillance sites supported by AFRIMS, the planned ILI-protocol will involve collection of respiratory samples that will be provided to the national surveillance system (through Provincial Health Departments) overseen by the Cambodian CDC. If resources permit, we will attempt local rapid testing and follow-up on-site testing by PCR in Battambang, with possible confirmatory testing at AFIOH in San Antonio, Texas. All results will be shared with the Cambodian MoPH and WHO. Local capacity will be built in areas such as specimen collection and transport, epidemiology, laboratory techniques, and AI recognition and responses. An assessment trip was made to Battambang and Phnom Penh to discuss preliminary plans with future partners in Cambodia and a proposal is drafted.

C. Department of Enteric Diseases, AFRIMS CY08 Research Efforts

1. Title of Research Project: Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand

    a. Investigators:

- Ladaporn Bodhidatta
- Umaporn Suksawad
- Ovath Thonglee
- Chittima Pitarangsi
- Boonchai Wongstitwilairoong
- Wilawan Oransathid
- Paksathorn Puripanyakom
- Orapan Chiwaratanond
- Pimmnapar Neesanant
- Sasikorn Silapong
- Tasawan Singhsilarak
- Kaewkanya Nakjarung

b. Objectives:

Monitor diarrhea etiology and antimicrobial resistance of enteric pathogens at multiple sites within Thailand.

c. Methods:

Surveillance was conducted in children under 5 years of age in hospitals and regional laboratories in several sites of Thailand to include Chiangrai, Phitsanulok, Nakornratchasima,Trang and Bangkok. Stool samples were received from each participating site for on-site initial assessment followed by confirmatory tests and additional laboratory studies to include molecular studies and antimicrobial susceptibility testing at AFRIMS and Thai NIH, MOPH in Bangkok Thailand.

d. Results:

Protocol approval process and field laboratory setup and training were completed. Surveillance is being conducted in all 5 sites. Over 500 stool samples have been collected from regional sites and over 400 samples were collected in Bangkok. Campylobacter and rotavirus were identified as leading causes of acute diarrhea.

e. Future Plans: Continue study.

2. Title of Research Project: Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens

a. Investigators:

- Orntipa Sethabutr
- Pimmnapar Neesanant
- Sasikorn Silapong
- Kaewkanya Nakjarung
- Tasawan Singhsilarak

b. Objectives:

Develop and standardize realtime PCR assays for the detection and characterization of enteric pathogens including Shigella, Enterotoxigenic *E.coli* (ETEC), Campylobacter, Cryptosporidia, Cyclospora, Norovirus, Sapovirus and Rotavirus

c. Methods:

Based on literature review and best available sequence data, multiples sets of primers and probes were designed for each pathogen of interest. The sets were initially evaluated against cultured material. Selected sets of primers and probes were then tested against frozen stool samples collected and archived from multiple Department of

Enteric Diseases studies. Lower limits of detection for several sets of primers and probes were determined.

d. Results:

Probes and primer sets have been developed and evaluated for Shigella, ETEC, Campylobacter, Cryptosporidia, Cyclospora, Norovirus, Sapovirus and Rotavirus. Standardized and validated methods were applied to routine detection of pathogens in clinical specimens. During CY2008, over 2,000 frozen clinical stool specimens received from multiple study sites were processed and investigated for Norovirus, Sapovirus and Rotavirus infection. Rotavirus and Norovirus were the major pathogens detected by real time PCR.

e. Future Plans:

Transfer validated and evaluated assays of ETEC, Shigella and Cryptosporidium to JBAIDS platform. (b) Further characterize the genotype of Noro/Rotaviruses by PCR and nucleotide sequencing.

3. Title of Research Project: Characterization of Enteric Pathogens Isolated from Children and adults in the Maldives

a. Investigators:

- Ladaporn Bodhidatta
- Boonchai Wongsatitwilairoong
- Apichai Srijan
- Pimmnapar Neesanant
- Sasikorn Silapong
- Kaewkanya Nakjarung
- Tasawan Singhsilarak

b. Objectives:

Determine diarrhea etiology and antimicrobial resistance of enteric pathogens from children and adults with diarrhea in Male and 2 regional hospitals in the Maldives

c. Methods:

The study protocol was approved by both the US and local authorities. Additional equipment and supplies were provided to the microbiology laboratories at the Indira Gandhi Memorial Hospital in Malé and 2 other regional hospitals. After obtaining informed consent, stool specimens were collected from diarrhea cases on presentation. Initial stool examination, culture and identification of enteric pathogens were performed in Maldives. Confirmatory tests and additional studies to include molecular studies and antimicrobial susceptibility testing were conducted at AFRIMS in Bangkok.

d. Results:

A total of 57 adults and 73 children with diarrhea were enrolled in a one month cross sectional study during August - September 07. The common pathogens isolated and initially confirmed from adults with diarrhea were Rotavirus, *Aeromonas* and *V.parahemolyticus*. The common pathogens isolated and initially confirmed from children with diarrhea were Rotavirus, EPEC and *Campylobacter.* Norovirus, Rotavirus and giardia/cryptosporidium were detected by a realtime RT PCR and EIA from frozen stool samples completed during CY08. Norovirus was identified in 41% of children and 7% of adults with diarrhea. Rotavirus was identified in 18% of children and 15% of adults with diarrhea. Giardia/cryptosporidium was found in less than 1%.

e. Future Plans: Data analysis and manuscript preparation for publication

4. Title of Research Project: Capsule genotyping system for *Campylobacter jejuni* and Sequencing of Capsule Locus of *C. jejuni* Type Strain HS O:42.

a. Investigators:

- Oralak Serichantalergs
- Piyarat Poothong
- Panida Nobthai

b. Objectives:

1. To validate PCR assays to detect capsule genotype among *C. jejuni* isolatesfrom Thailand using ten primer sets of capsule genotypes O: 1, O: 2, O: 3, O: 4, O: 6, O: 10, O: 15, O: 23/36, O: 41 and O: 53.

2. To sequence the unknown capsule locus of *C, jejuni* type strain.

c. Methods:

Ten primer sets of capsule genotypes (O: 1, O: 2, O: 3, O: 4, O: 6, O: 10, O: 15, O: 23/36, O: 41 and O: 53) were designed at NMRC. AFRIMS tested these primers against 103 Thai *C. jejuni* isolates of known Penner serotypes (performed by Canadian Lab) by PCR. The PCR assays were validated.

Different primer sets for capsule locus were designed at NMRC. Genomic DNA of *C. jejuni* Penner type strain HS 42 was amplified for conserved and variable part of capsule locus by long PCR assay. Amplified products were subjected for sequencing using TOPO subcloning kits.

d. Results:

1. Ten primer sets of capsule genotypes have been tested with 103 strains of C. jejuni isolates. Primer sets successfully discriminated most of Thai strains at the level of penner complexes were primers for HS O:1, O:2, O:3, O:4, O:23/36. The HS O:10, O:53 primer sets correctly identified strains of HS10 and HS 53.

Additionally, primer sets for HS O: 23/36 and HS O: 2 also discriminated additional C. jejuni isolates from the same PFGE cluster that shared closed similarities. Relationship of the probes and capsules types needed for further evaluated.

2. For capsule clocus sequencing of type strain HSO:42, primer set for the conserve site of capsule locus (*kps*C-R and *hdd*A-L) or *kps*C-Rand *dmh*A-L could amplified PCR product around 8-9 Kb. TOPO shortgun subcloning kit generated 75 clones. These 75 clones have been submitted for sequencing by Macrogen, Korea. The sequences showed good quality. All 75 sequences were edited, aligned and assemble. Blast search showed this sequence (conserved site) of capsule was similar to HSO: 41 type strain from previous publication. Only 4 gaps were generated and 4 PCR primers of these gaps were designed by Dr. Poly at NMRC. PCR of these 4 primer sets could amplify products of 700bp, 600 bp, 500 bp, and 400 bp. These PCR products were sequenced and all sequences assembly can fill to the gap. Sequencing of the conserved part of capsule of HS: O: 42 was completed.

e. Future Plan

Continue to collaborate with NMRC to evaluate capsule primer sets with other Thai C. jejuni strains and continue to sequence the variable part of capsule locus of type strain HS O:42 and other type strains.

5. Title of Research Project: Adaptation of the Established Rhesus Monkey Intragastric Challenge Model of Shigellosis to Study WRSd1 a Live Attenuated *Shigella dysenteriae*-1 Vaccine Candidate

a. Investigators:

- Dilara Islam, Ph.D
- Nattaya Ruamsap
- Ajchara Aksomboon
- Patchariya Khantapura
- Chittima Pitarangsi
- Boonchai Wongstitwilairoong
- Wilawan Oransathit
- Paksathorn Puripanyakom
- Sawat Boonnak
- Songmuang Piyaphong
- Kaewkanya Nakjarung

- Tasawan Singhsilarak
- DVM Montip Gettayacamin (Vet-Med, AFRIMS)
- Dr. Malabi Venkatesan (WRAIR)

b. Objectives:

The primary objectives of these protocols are:

i) To evaluate safety, by monitoring the presence and severity of physical signs and symptoms in monkeys following immunization with multiple doses of WRSd1;

ii) To evaluate protective efficacy after immunization with multiple doses of WRSd1 by challenging immunized monkeys with S. dysenteriae-1 1617 strain (parent strain of WRSd1) compared to challenging control monkeys with S. dysenteriae-1 1617 strain;

iii) To evaluate the immune responses and inflammatory responses (by measuring cytokines) in blood, stool, colonic lavage and colonic biopsy.

iv) To monitor shedding of WRSd1 and 1617 strain by standard fecal culture procedure and by PCR assay.

The doses are:

i) Immunized group: Immunization on days 0, 3 and 6 with 2×10^{10} cfu of WRSd1 & challenge on day 37 with 10^{10} cfu of *S. dysenteriae*-1 1617 strain;

ii) Control group: no immunization and challenge on day 37 with 10^{10} cfu of *S. dysenteriae*-1 1617 strain

c. Methods:

The study protocol was approved by the AFRIMS' IACUC. In order to establish the animal model of *S. dysenteriae* 1 infection, various specimens were taken for analysis, including, blood, stool, colonic lavage and colonic biopsies. Various clinical, immunologic, bacteriological, and histological tests were conducted on animal specimens from all monkeys. Serum IgA, IgG and IgM antibody titers, antibody secreting cells, fecal secretory-IgA and fecal cytokines were measured at different time points of the study.

All monkeys were monitored by direct observation for 30 minutes (after immunization and challenge) for occurrence of immediate adverse reactions. Also, beginning on day (-3) and continued 7 days after last immunization and after challenge or until all animals are clinically normal the monkeys were closely observed and scored twice daily using a clinical observation report (VM Form B20). Animals showing any signs of clinical disease were removed from the cage daily, physically examined by a veterinarian, and provided appropriate veterinary medical care.

d. Results:

Our result showed that WRSd1 vaccine is effective. Four of the five immunized animals were protected. No immunized animals developed dysentery but one animal developed severe vomiting with blood and died acutely; this might be the effect of Shiga toxin, as WRSd1 vaccine candidate can not protect against Shiga toxin, as the whole toxin gene is deleted in WRSd1. Five control monkeys developed diarrhea with blood and/or mucus, vomiting, dehydration, anorexia and depression within 1 to 3 days after challenge. One control monkey died acutely despite antibacterial treatment and supportive therapy.

Clinical result in both group of monkeys are shown in Table (below).

Monkey Groups	Clinical observation: (After immunization)	Clinical observation: (After challenge)
Group 1 (Immunization)	Anorexia (1/5)	• Soft stool with mucous & salivation, died with bloody vomiting content: (1/5) • Soft stool: (1/5) • Anorexia: (4/5)
Group 2 (Control)	No Immunization	• Watery stool with mucous and blood: (1/5; died) • Loose stool with mucous and severe vomiting: (3/5) • Severe vomiting with muco-bloody content: (1/5)

e. Future Plans:

The next following study will be a GLP study, where we will evaluate live, attenuated oral WRSd1 variant vaccine candidates, yielding information to further improve protective efficacy, reduced reactogenicity, and increased immunogenicity and compare with parent WRSd1 vaccine.

6. Title of Research Project: The Production of Antisera in Nonhuman Primates Against Live *Shigella Sonnei* 53G Strain

a. Investigators:

- Dilara Islam, Ph.D
- Nattaya Ruamsap
- Ajchara Aksomboon
- Patchariya Khantapura
- Chittima Pitarangsi
- Boonchai Wongstitwilairoong
- Rawiwan Imerbsin (Vet-Med)

b. Objectives:

The objective of this protocol was to produce antisera against *S. sonnei* 53G by intragastric immunization of rhesus monkeys. These antisera will be used as reference sera in ELISA procedures. *S. sonnei* 53G antisera is not commercially available; therefore, we must create the antisera for use as reference sera in ELISA for WRAIR HURC Protocol# 1259.

c. Methods:

A total of 6 rhesus monkeys, (2 groups of 3 each) were randomly assigned regardless of sex to Group 1 and Group 2. Initially Group 1 monkeys were immunized by intragastric administration of S. sonnei 53G strain with a dose of 2×10^8 cfu in 20 ml sterile PBS on study day 0 and boosted on day 21. Likewise Group 2 monkeys were immunized by intragastric administration of S. sonnei 53G strain with a dose of 2×10^9 cfu in 20 ml sterile PBS on study day 0 and boosted on day 21. Due to low titers in group 1, these monkeys were rechallenged with two additional doses of S. sonnei 53G strain at a dose of 2×10^9 cfu in 20 ml sterile

Experimental Groups and Immunizations Summary

Monkey groups	Study Days	Immunizations
Group 1 (3 monkeys)	0 and 21	Immunization with *S. sonnei* 53G strain at 2×10^8 cfu dose
Group 2 (3 monkeys)	0 and 21	Immunization with *S. sonnei* 53G strain at 2×10^9 cfu dose

Blood and stool samples were collected.

d. Results:

The Protocol was completed and reference sera generated.

e. Future Plans: N/A.

7. Title of Research Project: "Exempt" Human Use Protocol: Establish ELISA Reference Sera to be Used for Protocol "Establishment of a *Shigella sonnei* Challenge Model for Evaluation of Future Vaccine Candidates"

a. Investigators:

- Dilara Islam, Ph.D
- Nattaya Ruamsap
- Ajchara Aksomboon
- Patchariya Khantapura

b. Objectives:

The objective of this study was to generate reference plasma/sera from human or animal for use in Enzyme-linked immunosorbent assay (ELISA) to measure antibody responses against Shigella sonnei LPS and/or S. sonnei protein antigens (Ipa proteins).

c. Methods:

SOP: ETR-IM-000 was followed to select and screen samples to be used as positive and negative control sera. Serum samples from individuals with stool culture confirmed for *S. sonnei* were selected for positive reference sera and sera from Cobra Gold study with low titer against *S. sonnei* LPS were selected for negative reference sera. Titers (IgA/IgG/IgM) of individual serum sample was tested against. *S. sonnei* LPS by ELISA following SOP: ETR-IM-000. Purified *S. sonnei* LPS is purchased from Commonwealth Biotechnologies, Inc. USA. Positive reference sera: Depending on the obtained optical density (OD) value of individual tested serum sample; serum samples were selected to make the pool so the OD of the pool is not lower than 0.40. The expected OD value should be ≥ 0.40 for the pooled positive reference sera at dilution not lower than 1:100. Negative reference sera: A minimum of 5 samples with individual OD value between 0.1-0.2 (for all Ig classes) were pooled for use as negative reference sera.

d. Results:

Pooled sera created and subsequently used a reference reagent.

e. Future Plans: N/A.

8. Title of Research Project: Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Nepal

a. Investigators:

- Ladaporn Bodhidatta
- Apichai Srijan
- Paksathorn Puripanyakom
- Wilawan Oransatit
- Orapan Chivaratanond
- Sawat Boonnak
- Boonchai Wongstitwilairung

b. Objectives:

Monitor diarrhea etiology and antimicrobial resistance of enteric pathogens at 3 sites in Kathmandu and Bharatpur, Nepal.

c. Methods:

Three human use protocols approved by both the U.S and Nepal authorities Studies have been conducted in children in Kanti Children Hospital in Kathmandu and in Bharatpur Hospital, Bharatpur and in adults in Sukraraj Tropical Infectious Disease Hospital, Kathmandu. Several visits were made by the Principal Investigator, as well as nursing and laboratory staff prior to study initiation.

Microbiology laboratory, media preparation, ELISA and QA/QC capability have been established at WARUN. Laboratory procedures for specimens collected in Kathmandu were performed at WARUN. Stool samples received at Bharatpur Hospital were initially assessed on site and sent for confirmatory tests and additional laboratory studies at WARUN. A new microbiology laboratory was completed at Bharatpur Hospital by space renovation. Onsite trainings were conducted. AFRIMS will perform molecular studies and antimicrobial susceptibility testing of the specimens collected from all sites.

d. Results:

Surveillance has been conducted in all 3 sites. Over 2500 stool samples have been received from subjects with and without diarrhea. The leading pathogens significantly identified in children with acute diarrhea were rotavirus (>20%), Enterotoxigenic *E.coli* (ETEC) and *Shigella*. In adults with acute diarrhea, ETEC, *Campylobacter* and *Shigella* were detected as major pathogens..

e. Future Plans: Continue study.

9. **Title of Research Project:** Establishment of a *Shigella sonnei* Challenge Model for Evaluation of Future Vaccine Candidates

a. Investigators:

- Ladaporn Bodhidatta
- Dilara Islam, Ph.D
- Umaporn Suksawad
- Apichai Srijan
- Orntipa Sethabutr
- Nattaya Ruamsap
- Ajchara Aksomboon
- Patchariya Khantapura
- Boonchai Wongstitwilairoong
- Tasawan Singhsilarak
- Kaewkanya Nakjarung
- Sasikorn Silapong

b. Objectives:

To identify the optimal challenge dose of *Shigella sonnei* 53G that will elicit clinical diseases in at least 70% of the healthy Thai volunteers.

c. Methods:

The study protocol was approved by 2 Thai IRBs (Ministry of Public Health and Faculty of Tropical Medicine) and the U.S IRB (HSRRB) with the main purpose to establish a Shigella sonnei challenge model in Thai adults. This will serve as the target attack rate for follow-on efficacy trials of future vaccine candidates against *Shigella sonnei*.

A sequential evaluation of three challenge doses of *S. sonnei* 53G at 100, 400 and 1600 cfu has been proposed. A group of 12 healthy Thai adult volunteers will be admitted to the Vaccine Trial Center (VTC) of the Faculty of Tropical Medicine, Mahidol University for a period of 8 to 11 days. Baseline stool, blood, serum, and urine samples will be taken. Volunteers will be orally challenged with *S. sonnei* 53G after a sodium bicarbonate solution to neutralize stomach acidity and will be closely monitored for symptoms and signs of shigellosis. Stools will be collected for clinical characterization and for culture and PCR, blood will be collected for immunology assays and clinical laboratory testings. After five days (120 hours) post challenge, volunteers will be treated with oral ciprofloxacin for 3 days unless specific early antibiotic treatment criteria are met. Volunteers will be hospitalized until they are symptom-free and 2 sequential stool cultures are negative for *S. sonnei* and 6 doses of oral ciprofloxacin therapy are completed. After discharge from the facility, volunteers will be asked to come for follow up visits on on Day 14 and Day 28. A telephone visit on Day 42 will also be made to monitor late complications.

d. Results:

The first cohort of 12 healthy Thai adults volunteers were challenged with 93 cfu of *S.sonnei* 53G on 10 September 07. The disease of fever, diarrhea, and/or dysentery was observed in 3 out of 12 volunteers (25%). The adverse events were generally as a result of the challenge. One serious adverse event with an elevation of total bilirubin, which returned to normal during follow up, was reported in one volunteer. Study medical monitors were in agreement that the data from the first cohort did not meet the target attack rate and no safety concern and the second cohort was scheduled.

The second cohort of 12 healthy Thai adults volunteers were challenged with 440 cfu of *S.sonnei* 53G on 15 Jan 08. The disease was observed in 6 out of 12 (50%). The adverse events were generally as a result of the challenge. No serious adverse event was observed during the admission and follow up.

Since the target attack rate was still not achieved in the second cohort and there was no safety concern, the third cohort of 12 healthy Thai adults volunteers were

challenged with 1680 cfu of *S.sonnei* 53G on 02 April 2008. The disease was observed in 9 out of 12 volunteers (75%) and exceeded the target attack rate of 70%. Dysenteric stool was observed in 9 out of 12 volunteers (75%). Excretion of *S. sonnei* in at least one stool was observed in 11 out of 12 volunteers (92%). Adverse events were generally as a result of the challenge. One serious adverse event was reported in one volunteer who had an increased total bilirubin, classified as grade IV according to CBER's guideline, with no other associated signs and symptoms at Day 14. The total bilirubin level returned to normal without any treatment.

Data management of the study has been conducted by the Center of Excellence for Biomedical and Public Health Informatics, Mahidol University.

e. Future Plans: Data analysis and manuscript preparation for publication

10. Title of Research Project: Surveillance of Respiratory Pathogens in Patients Attending Royal Thai Army Hospitals

a. Investigators:

- Jariyanart Gaywee (RTA)
- Narongrid Sirisopana (RTA)
- Chirapa Eamsila (RTA)
- Pochaman Watcharapichat (RTA)
- Thippawan Chuenchitra (RTA)
- Khin Saw Aye Myint (Virology)
- Ladaporn Bodhidatta
- Pimmada Jeamwattanalert

b. Objectives:

To diagnose respiratory pathogens in patients attending Royal Thai Army hospitals and to provide influenza surveillance data to the WHO surveillance network

c. Methods:

Eligible patients will be identified in the outlined study hospitals by study team staff based on the eligibility criteria. Eligible subjects will be presented the study information sheet and asked to participate in the study. If they agree, written informed consent will be obtained by study team staff medical history collected as outlined in the Demographic/Clinical Form by study team staff. Additional diagnostic tests and any treatment decisions will be solely made by the attending physician, in accordance with local standards and the available medical data.

d. Results:

Of the samples collected at the six sites from a total of 982 volunteers, 845 samples collected have been influenza negative by on site rapid testing; 137 were positive (91 influenza A & 45 influenza B & 1 both influenza A & B). More comprehensive PCR testing at AFRIMS on an initial 38 samples found 34 negative and 4 positive (4 influenza A/H3). Further testing on these samples in pending.

e. Future Plans:

During the coming year, we plan to continue enrollment of volunteers at all sites and amend the protocol to expand enrollment to additional sites.

D. Department of Veterinary Medicine AFRIMS FY08 Research Accomplishments

1. Title of Research Project: Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta)/Plasmodium cynomolgi* Relapsing Malaria Model

a. Investigators:

- Dr. Montip Gettayacamin
- Ms. Pranee Hansukjariya
- Dr. Jetsumon Prachumsri
- Mr. Srawuth Komchareon

b. Objectives:

1. Use the rhesus monkey/*P. cynomolgi* model to determine the effectiveness of new causal prophylactic and radical curative compounds which are being synthesized and developed by the US Army antimalarial drug development program.

2. Use the rhesus monkey/*P. cynomolgi* blood-stage malaria model to evaluate new antimalarial compounds for their blood schizonticidal activity.

c. Methods:

Malaria is one of the most important parasitic diseases worldwide. Traditional treatment for malaria includes drugs used to prevent disease (prophylaxis) and to cure the infection (therapeutic). Antimalarial drug screening in the rhesus monkey model is very effective for making comparisons between drugs. It is fairly rapid, relatively inexpensive, and makes reliable predictions of how drugs will in act in man. Antimalarial drug screening in the rhesus monkey has played a key role in the development of every antimalarial drug licensed in the the US for the past 30 years. This model provides a mechanism to identify effective new drugs for the enhanced prevention and treatment of malaria infections.

d. Results:

One experiment was conducted in this fiscal year using monkeys. Four Imidazolidinedione (IZ) derivatives (WR301855, WR299666, WR301795) and a pyrimidine derivative (WR301798) were screened for causal prophylactic activity and radical curative activity. WR301855 and WR301795 had tissue schizonticidal activity to delay parasite patency and relapse. Another compound (WR283205) was tested at 3 dose regimens for radical curative activity at the primary infection, 1st or 2nd relapses. Tissue schizonticidal activity to delay was found at 30 mg/kg for 3 days intramuscularly or 7 days orally. The other compounds had no activity.

e. Future plans:

We anticipate conducting at least two experiments over the next year.

2. Title of Research Project: Care and Maintenance of Rhesus Monkeys *(Macaca mulatta)* and Management of Breeding Colonies

a. Investigators:

- Dr. Montip Gettayacamin
- Mr. Srawuth Komcharoen

b. Objectives:

Maximize the production of specific pathogen-free rhesus monkeys in the USAMC-AFRIMS production colony, using the best and most humane husbandry care, maintenance procedures, veterinary care, and disease surveillance and environmental enrichment procedures available.

c. Methods:

USAMC-AFRIMS maintains a breeding colony of rhesus macaques using a closed colony system. Approximately 150 rhesus monkeys are used in the breeding program. Two types of breeding are managed: compatible male and female pairs are housed in special paired-type caging, and multiple harem groups are established and maintained in large gang cages. Harems consist of one breeding sire and 5-15 adult females. Newborn monkeys are weaned at approximately 6 months of age, and then are reared to adulthood in gang cages with other weanlings. All colony primates are tested routinely for the presence of infectious diseases that pose a threat to either the health of the colony or to personnel working with the primates. Humane use of the animals is assured by the intense oversight of the Institutional Animal Care and Use Committee. Veterinary and technical care is extensive and continuous. Whenever possible, animals are re-utilized in multiple protocols in order to optimize the use of this limited and essential resource.

d. Results:

Fifty-one (51) baby rhesus macaques were born in the colony in the last year. To increase the genetic diversity of the USAMC-AFRIMS rhesus colony, new imported male rhesus monkeys were successfully transported from the U.S. to Thailand on October 20, 2008.

e. Future plans:

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free nonhuman primates to support USAMC-AFRIMS research needs. Maintain and expand the colony by adding the 10 new breeding males and increasing the number of paired housing cages.

3. Title of Research Project: Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance/ Quality Surveillance Program

a. Investigators:

- Dr. Montip Gettayacamin
- Ms. Anchalee Tungtaeng

b. Objectives:

Maintain a breeding colony of specific pathogen-free laboratory rodents to meet the scientific research needs of the USAMC-AFRIMS, using state-of-the-art knowledge, equipment, and facilities.

c. Methods:

USAMC-AFRIMS maintains breeding colonies of laboratory rodents to meet the needs of AFRIMS research. Using state-of-the-art equipment, knowledge, and facilities, production is matched to the anticipated needs of individual research projects. Extensive and thorough recordkeeping ensures that outbred strains remain outbred, and that inbred strains remain truly inbred. An extensive quality assurance/quality surveillance program, which includes serologic assessments as well as necropsy/histopathologic analysis, ensures that the colony produces only high-quality disease-free animals. When necessary, new breeder stock is procured from a reliable vendor. Veterinary and technical care is extensive and continuous.

d. Results:

Six thousand six hundred thirty-one (6,631) ICR mice (*Mus Musculus*) were produced and 2,980 mice were used for 6 active protocols. Five hundred and eight (508) hamsters were maintained for one protocol to maintain mosquito colonies. Quality

assurance procedure monitors the health status of the mice produced in the colony and hamsters purchased from a local vendor.

e. Future plans:

These breeding colonies will be continued and maintained in order to provide a cost-effective means of supply of specific pathogen-free rodents to support USAMC-AFRIMS research needs.

4. Title of Research Project: A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs

a. Investigators:

- Dr. Montip Gettayacamin
- Ms. Pranee Hansukjariya
- Ms. Anchalee Tungtaeng

b Objectives:

To evaluate potential antimalarial chemotherapeutic agents in the Blood-stage *P. berghei* ICR mouse model.

c. Methods:

The test system used for the determination of antimalarial activity of the compounds is a modification of the suppressive test known as the Thompson Test. Typically in this test, up to 22 groups of 5 mice are inoculated intraperitoneally (IP) with *P. berghei*-infected erythrocytes then treated with candidate drugs to determine the antimalarial activity. Infected erythrocytes are provided from donor mice. On experiment day 0, the donor mice are anesthetized and they were exsanguinated via cardiac puncture. The blood samples are pooled and the level of parasitemia is determined. The pooled blood is diluted with normal mouse serum to a concentration of 1×10^6 infected erythrocytes per an inoculum (0.1 ml). The groups of experimental and control mice are inoculated with the standard 0.1 ml infected blood on day 0. On day 3, 4, and 5 mice in each group are treated with either the candidate antimalarial drug at the determined dose or with vehicle alone, to serve as the negative control. The drug is administered orally (PO), subcutaneously (SC), intramuscularly (IM) or intraperitoneally (IP) up to two times daily, based on the unique pharmacokinetics of the test compound. Each experimental group receives a different dose level, with up to 5 different dose groups per compound. A standard antimalarial drug may be tested along with the candidate drug for structure-activity determination and for quality assurance of the model. Blood smears are obtained on the study days 0, 3, 6, 10, 13, 17, 20, 24, 27, 31, and if necessary at twice weekly intervals through day 60. Individual mouse body weights are taken on the study days 0, 3, 4, 5, 6, 7, 10, 13, 17, 20, 24, 27, 31, and if necessary at twice weekly intervals through day 60. Blood films are stained, examined by light microscopy, and the percent parasitemia determined. All mice are observed twice a

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day to assess their clinical signs. All mice with negative smears at 31 days (or 60 days) are considered cured.

d. Results:

A total of 18 compounds were tested in 7 experiments.

e. Future plans:

This mouse model for screening new candidate antimalarial compounds is very effective for making comparisons between drugs. It is rapid, relatively inexpensive, and makes reliable predictions of how drugs will act in higher mammalian hosts, including humans. This is a core capability of the USAMC-AFRIMS Department of Veterinary Medicine and will be maintained so that many more compounds can be tested.

5. Title of Research Project: *Plasmodium berghei - Anopheles dirus* Sporozoite - ICR Mice Malaria Model for Screening Exoerythrocytic Antimalarial Drugs

a. Investigators:

- Dr. Montip Gettayacamin
- Dr. Jetsumon Prachumsri
- Ms. Anchalee Tungtaeng

b. Objectives:

To evaluate potential causal prophylactic antimalarial agents in the *P. berghei* mouse exoerythrocytic (EE) model at AFRIMS.

c. Methods:

A model involves infecting mice with sporozoites harvested from infected Anopheline dirus mosquitoes on day 0. Generally, there are up to 11 groups of 5 mice per group or up to 55 animals in each experiment. Usually on day -1, day 0 (within 1 to 3 hours prior to the inoculation) and day 1, each mouse in each group receives a testing compound at the determined dose, frequency and route as in the amendment. Each compound may be tested at 1 to 5 doses. Control animals receive a same amount of vehicle without drug. Testing compounds are administered to the mice in the corresponding groups by oral (PO), subcutaneous (SC), intramuscular (IM), intravenous (IV), or intraperitoneal (IP) or transdermal routes for up to two times a day as outlined in the amendment. On day 0, all mice (control and experimental mice) are infected by a standard 0.1-ml dose of 1.0×10^5 *P. berghei* sporozoites by intravenous (IV) inoculation. Animal weights are obtained on study days –2, -1, 0, 6, 10, 15, 21 and 31 (or included day -3 if the compound administration is scheduled on day -2, or on the days the compound administration is scheduled as outlined in the amendment). Blood smear samples are obtained on days 4, 5, 6, 7, 10, 15, 21 and 31 post-inoculation. Mice are observed twice daily for clinical signs and mortality. Mortality

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and clinical signs are recorded. Animals showing combined clinical signs within the early endpoint criteria (unable to move or severe depression, reduced appetite, extreme pallor and ruffled hair) are euthanized by CO_2. On day 7 and afterward, animals with parasitemia exceeding 5% are humanely euthanized. Prior to euthanasia, blood smears are obtained for parasitemia determination. Mice survive on day 31 are euthanized by CO_2. All mice are necropsied. Mice with negative smears at 30 days are considered to be protected.

d. Results:

The protocol screened 74 compounds in 26 experiments.

e. Future plans:

This mouse model for screening new candidate antimalarial compounds is very effective and makes reliable predictions of new compounds and screens new antimalarial compounds against the exo-erythrocytic (liver stage parasites) before further testing in monkey malaria model. This core capability of the USAMC-AFRIMS Department of Veterinary Medicine will be maintained and many more compounds will be screened.

6. Institutional Animal Care and Use Committee

a. Personnel:

- Dr. Julie A. Pavlin, IACUC Chair
- Dr. Yvonne A. Van Gessel, Alternate Chair
- Dr. Sarah B. Hinds, Attending Veterinarian
- Dr. Montip Gettayacamin, Alternate Attending Veterinarian
- Dr. Rawiwan Im-erbsin, Alternate Attending Veterinarian
- Dr. Kriangkrai Lerdthusnee, Scientist 1
- Dr. Wantanee Ratanasak, Alternate Scientist 1
- Dr. Warawadee Nirdnoy, Scientist 2
- Dr. Ladaporn Bodhidatta, Alternate Scientist 2
- Dr. Chuanpis Ajariyakhajorn, Scientist 3
- Dr. Robert Paris, Alternate Scientist 3
- Dr. Pakitya Teja-isavadharm, Scientist 4
- Dr. Sathit Pichyangkul, Alternate Scientist 4
- Dr. Kyle Hathaway, Scientist 5
- Ms. Somporn Krasaesub, Statistician
- Ms. Pringsri Ingkaninun, Non-Affiliated/Non-Scientist
- Ms. Swalee Siriphol, Alternate Non-Affiliated/Non-Scientist
- SSG. Marc Bellaire, IACUC Coordinator
- Ms. Angwara Arinhamapan, IACUC Administrator

b. Objectives:

1. To support the animal research of USAMC-AFRIMS by providing oversight for the USAMC-AFRIMS animal care and use program.

2. To review all proposed animal research protocols.

3. To assure IACUC members are trained in current SOP's and issues related to managing a quality animal care and use program

c. Methods:

The IACUC is the self-regulating body for animal research on behalf of the iAAnstitute. In accordance with regulations, the USAMC-AFRIMS IACUC meets a minimum of once every six months and typically meets once a month. All new protocols are reviewed by a full committee. The IACUC monitors the animal care and use program by conducting thorough reviews of the program and inspections of animal facilities semi-annually. Particular attention is paid to the justification for the use of animals, unnecessary duplication of studies, alternatives to animal use, early endpoints, pain and distress procedures, and euthanasia. Additionally the IACUC upholds that appropriate, documented training for principal investigators, technicians, and staff is in place prior to the initiation of any animal study, to include proper occupational health and safety requirements. Once a study is underway, the IACUC may perform post-approval compliance monitoring to verify quality animal care is intact.

d. Results:

The Association for Assessment and Accredidation of Laboraotry Animal Care International (AAALAC) conducted its second triennial site visit to assess and determine accredidation status of the USAMC-AFRIMS Animal Care and Use Program. The site visit team was extremely impressed with the program; their assessment was "Continued Full Accreditation with No Suggestions". This is an outstanding outcome as less than 5% of all programs reviewed world-wide receive this assessment. The program was awarded "exemplary" status in the official notice for the second time in a row (the last site visit in 2005 had the same notation)

The IACUC held five full committee meetings in 2008. The IACUC supported 26 active protocols during 2008, six of which were approved during the calendar year. Several amendments (39) were reviewed and approved. Two semi-annual facility inspections and program reviews were completed and reviewed. A training seminar was conducted in cooperation with Mahidol University and the Thai Association of Laboratory Animal Science (TALAS) in addition to training scheduled during normal meeting periods, totaling nine hours. Additionally, three IACUC members attended the 2008 American Association of Laboratory Animal Science (AALAS) convention.

e. **Future Plans:**

USAMC-AFRIMS will have its third triennial Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALACi) site visit in March 2011. Continued facility maintenance and upgrades are planned to maintain an exemplary program. Continued accreditation is expected, underscoring USAMC-AFRIMS' commitment to quality animal care.

E. Department of Virology, AFRIMS CY08 Research Accomplishments

1. Title of Research Project: Prospective Study of Dengue Virus Transmission and Disease in Primary Schools and Villages in Kamphaeng Phet, Thailand

a. **Investigators:**

1. Principal Investigators:

- In-Kyu Yoon, LTC, MD, MC (USAMC-AFRIMS)
- Suwich Thampolo, MD, MPH (Dengue Office, Division of Vector-Borne Diseases, Ministry of Public Health (MOPH))
- Chusak Pimgate, M.D., MC (USAMC-AFRIMS)

2. Associate Investigators (by institution):

Armed Forces Research Institute of Medical Science (AFRIMS):

Department of Virology

- Robert V.Gibbons, M.D.,MPH, LTC, Chief
- Richard G.Jarman, B.S., Ph.D, Chief, Laboratory Operations
- Ananda Nisalak, M.D., Consultant in Arbovirology
- Charity Ann M. Ypil-Butac, M.D., Consultant in data analysis
- Butsaya Thaisomboonsuk, Ph.D., Head, Arbovirology Section
- Chonticha Klungthong, Ph.D., Head, Molecular Research Section

Department of Entomology

- Jittawadee Murphy, Ph.D., MAJ, Chief
- Thanyalak Fansiri, Entomology Study Coordinator, Mosquito Biology Section

Thai Ministry of Public Health (MOPH):

- Supamit Chunsuttiwat M.D., Senior Expert in Preventive Medicine, Disease Control Department, Ministry of Public Health

Institute of Urology and Nephrology, University College London, The Middlesex Hospital

- Henry A. F. Stephens, Ph.D.,
  Clinical Scientist and Head of Tissue Typing

Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School (UMMS):

- Anon Srikiatkhachorn, M.D., Assistant Professor
- Daniel H. Libraty, M.D., Assistant Professor
- Alan L. Rothman, M.D., Associate Professor
- Sharone Green, M.D., Associate Professor
- Francis A. Ennis, M.D., Director

Department of Entomology, University of California, Davis:

- Thomas W. Scott, Ph.D., Professor of Entomology and Director, Davis Arbovirus Research Unit
- Amy C. Morrison, Ph.D., Assistant Research Entomologist

Department of Geography, San Diego State University:

- Arthur Getis, Ph.D., Stephen and Mary Birch Chair of Geographical Studies

b. Objectives:

The goal of the proposed study is to identify those factors that have the strongest influence on determining the early events in acute DV infections, and the eventual clinical manifestations of disease. An equally important goal is to characterize protective immune responses (e.g. CD4$^+$ and CD8$^+$ T-cell responses, neutralizing antibody responses) as we have found that low levels of pre-existing neutralizing antibodies to a subject's own infecting virus isolate do not necessarily protect from symptomatic DV infection. We plan to prospectively identify host-specific factors (e.g., pre-existing memory T and B cell responses to DV, HLA genetic polymorphisms, viral burden and replication in the host), virus-specific factors (e.g. DV serotype, serotype infection sequence), and environmental factors (e.g. mosquito population patterns, mosquito viral burden) for asymptomatic and symptomatic secondary DV infections, particularly severe infections (DHF/DSS). Multi-year investigations are crucial to this study due to the year-to-year variations in the incidence and prevalence of circulating serotypes. An improved understanding of the correlations between the host, viral, and environmental factors and dengue disease severity will contribute to DV vaccine development and testing.

c. Study Specific Hypotheses:

1. Subjects with pre-existing neutralizing dengue antibodies above a definable threshold will be protected from DV infection or severe disease on subsequent exposure to virus.

2. The frequency of pre-existing CD4$^+$ and CD8$^+$ T-cells and their specific cytokine responses to stimulation with DV antigens will correlate with disease severity (protection or enhancement) and the plasma viral RNA levels measured in secondary DV infections.

3. Specific serotype sequence combinations of DV infections will elicit qualitatively and quantitatively distinct immune responses associated with illness of varying severity.

4. Higher viremia levels will be seen in secondary DEN-2 and DEN-4 virus infections in subjects with higher levels of *in vitro* antibody-dependent enhancing capability of pre-illness blood samples.

5. DV infection rates will cluster in households around a DV-infected index case and a correlation will exist between the number of susceptible contacts, and associated mosquito density, and mosquito infectivity (viral RNA levels).

6. DV disease severity will correlate with peak plasma viremia levels and associated mosquito density and mosquito infectivity (viral RNA levels).

7. Genes encoded within the human MHC, the NK killer inhibitory receptor (KIR) gene complex on chromosome 19, and the Fc gamma receptor gene complex on chromosome 1 influence the susceptibility, severity and resistance to primary and secondary DV infections.

d. Methods: In this study, we:

i) Continue the successful prospective, school-based, study platform to study dengue epidemiology in primary school children in KPP province, and

ii) Conduct a village-based, cluster surveillance study.

(a) This is a prospective school-based study of 2,000 children, which began in 2003 and will end in January 2008. Students in K2 to grade 6 are recruited and enrolled into the study. Baseline demographics are recorded and study numbers assigned. Each subsequent year, new K1-Grade 5 students are newly enrolled. Students are followed until they are either disenrolled, withdrawn by their parent/guardian, graduate from Grade 6 or when the study ends. Every year, plasma (PBMCs for Dengue Season 1 only) is collected from the entire cohort at the beginning of the surveillance period (June). Plasma and PBMCs are collected from the entire cohort at the end of the surveillance period (January). The hemagglutination inhibition (HAI) assay is performed on paired sera from the beginning and end of the surveillance

period to assess for flavivirus seroconversion. Plasma and PBMCs obtained at the end of the surveillance period in January serve as pre-illness samples in subjects who have a DV infection that same calendar year.

During the active surveillance period extending between June and November, those children who are absent from school (or who report ill to the teacher), will be evaluated either by a village health worker or AFRIMS nurse using a questionnaire and oral temperature measurement. Any child who has a documented fever (temperature ≥ 38 C) or reports illness with subjective fevers during the prior 7 days, is transported to the Public Health Office (PHO) where a public health nurse will do an evaluation. An acute blood specimen will be drawn. The child will be referred to the hospital at the discretion of the public health nurse. About 14 days later, an AFRIMS nurse visits the child to administer another questionnaire and to draw a convalescent blood specimen. The acute and convalescent specimens are evaluated by the AFRIMS dengue/JE IgM/IgG ELISA and HAI. The acute specimen will be evaluated further by dengue RT-PCR (and virus isolation techniques).

(b) Cases 'triggering' a cluster investigation are identified between Monday and Thursday of each week during the School-Based Component active surveillance period. Most specimens from acutely ill children arrive at the field station laboratory by 3pm each day. Upon arrival of the specimen, the database is reviewed to assess whether the child meets all index case inclusion and exclusion criteria. The field teams are notified of a possible case. The DV RT-PCR result (positive or negative) will normally be available by 11AM the following morning. No more than 30 positive and 30 negative clusters (as defined by the RT-PCR result of the index case) will be initiated in any given year. Once triggered, an Advance Team composed of a nurse and an entomological team supervisor visits the village and begins the consent form process. The exact location of all houses in each participating village has previously determined using a Global Positioning System (GPS) unit. Data points will be used to construct a digital map which will enable the team to precisely identify houses located within 100 meter radius of the index case and rapidly assess the likelihood of enrolling a minimum of 10 contacts. Once at least 10 contacts have been consented, the field teams will be dispatched to the village where the consent form process will continue. A clinical nurse will review the consent form, answer questions, address parental concerns, and obtain informed consent from the parents of susceptible contact children (ages 6 mo-15 yrs) residing within a 100 meter radius of the index household. Following the acquisition of parental consent, blood samples will be collected from 10-25 contacts. Those parents (and children) who are unavailable to be consented (and bled) are visited that same evening or the following morning. The clinical team will return to these homes approximately 5, 10 and 15 days after the initial visit to perform clinical assessments. The children bled on day 0 (initial specimen) are re-bled on approximately day 15 (follow-up specimen). DV RT-PCR will be performed on all acute specimens. If the day 15 blood is positive RT-PCR we will go out to draw blood on day 30 for doing ELISA. Dengue IgM/IgG ELISAs are performed on paired initial and follow-up specimens.

An entomological team collects mosquitoes, administers questionnaires, and performs insecticide spraying within the pre-determined meter radius of the index

household. Another entomological team will collect mosquitoes but not perform insecticide spraying around the classroom and school bathroom areas of the index case.

   e. **Results**:

   1. School cohort study

   The number of subjects approved for the school-based component was approximately 2000 at any time during each dengue surveillance season not to exceed a cumulative total of 4000 during the life of the study. In the study, there were 2095, 2088, 2086 and 2060 at the beginning of each dengue season in 2004, 2005, 2006, and 2007 respectively. The cumulative total of subjects enrolled in the school component during the entire study was 3526. The total number of subjects who withdrew from the study during all 4 years was 183. The major reason for withdrawal was relocation out of the study area. Thirty-three subjects withdrew due to objections to blood draws.

   During the 4 years of active surveillance (2004-2007), 2606 acute illnesses with fever on evaluation or report of fever within the prior 7 days underwent blood draws. Of these illnesses, 186 were serologically positive for dengue (12 acute primary dengue infections, 168 acute secondary dengue infections and 6 recent secondary dengue infections) out of which 148 were dengue PCR positive. DEN-1 was the most common serotype making up 47% of PCR positive cases. Forty children from the school component were hospitalized with confirmed dengue: 31 with dengue fever, 3 with DHFI, 3 with DHFII, and 3 with DHFIII. All hospitalized subjects were eventually discharged with complete recovery.

   2. Village-based study

   During the entire study, a total of 103 cluster investigations were performed: 51 positive clusters (involving 816 child contacts) based on dengue PCR positive index cases and 52 negative clusters (involving 783 child contacts) based on dengue PCR negative index cases. A total of 3182 blood specimens were drawn.

   During the entire study, there were a total of 51 positive clusters and 52 negative clusters. Between day 0 and day 15 evaluations, 116 of 816 contacts within the positive cluster investigations were serologically confirmed to have dengue infection of which 42 were dengue PCR positive on day 0. Six of 783 contacts within the negative cluster investigations were serologically confirmed to have dengue infection of which 2 were dengue PCR positive on day 0. In the positive clusters, there were 22 acute primary dengue infections, 80 acute secondary dengue infections and 14 recent dengue infections between day 0 and 15. In the negative clusters, there was one acute primary dengue infection and 5 acute secondary dengue infections between day 0 and 15. On day 15, 11 contacts within the positive clusters were dengue PCR positive (6 DEN-1 and 5 DEN-4) while one contact within the negative clusters was dengue PCR positive (DEN-4). Seven subjects from all the cluster investigations were hospitalized with confirmed dengue: 5 with dengue fever, 1 with DHFI and 1 with DHFII. All seven were discharged from the hospital with complete recovery.

As part of the entomological study, 24 of 3238 Aedes aegypti mosquitoes collected from cluster investigations were positive by dengue PCR (eleven DEN-1, three DEN-2, two DEN-3 and eight DEN-4). Twenty-three of these positive mosquitoes were collected from positive clusters and the dengue serotypes of these mosquitoes were the same as that of their respective positive index cases. Only one positive mosquito was from a negative cluster.

f. Future Plans:

No further contact with study subjects will take place. The human use portion of the protocol is complete. Data analysis is ongoing; presentations and manuscripts are being formulated to be presented at various international venues and journals.

2. Title of Research Project: The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue

a. Investigators:

1. Principal Investigators:

- Siripen Kalayanarooj, MD (Queen Sirikit National Institute of Child Health, Bangkok)
- Robert V. Gibbons, MD (USAMC-AFRIMS)

2. Associate Investigators:

- Ananda Nisalak, MD (USAMC-AFRIMS)
- Pra-orn Supradish, MD (QSNICH)
- Anchalee Krautrachue, MD (QSNICH)
- Lawan Wongtapradit, MD (QSNICH)
- Narong Nithipanya, MD (QSNICH)
- Warangkana Ratanaprakarn, MD (QSNICH)
- Anon Srikiatkhachorn, MD, Assistant Professor (UMMS)
- Daniel H. Libraty, MD, Assistant Professor (UMMS)
- Irene Bosch, PhD, Assistant Professor (UMMS)
- Alan L. Rothman, MD, Associate Professor, (UMMS)
- Sharone Green, MD, Associate Professor, (UMMS)
- Francis A. Ennis, MD, Director (UMMS)
- Henry A. F. Stephens, Ph.D., Clinical Scientist and Head of Tissue Typing, (University College London)

b. Objectives:

To identify the immunopathological mechanisms of dengue hemorrhagic fever (DHF), to analyze differences between DHF resulting from primary versus secondary

infections, to identify a sensitive method for detection of plasma leakage, and to characterize the dengue specific T cell response. The project encompasses studies from 2003 to 2008.

c. Study Specific Objectives:

1. Identification of clinical parameters which will predict clinical severities.

2. Evaluate the accuracy of sequentially measured semi-quantitative d-dimer assay, as compared to standard clinical parameters, at predicting the clinical progression to severe clinical dengue.

3. Determine if ultrasound or interstitial fluid albumin levels can predict early plasma leakage and shock. The ability to detect these shifts early in disease progression may help in prediction algorithms for DHF and permit early intervention with new therapies in the at-risk population;

4. Analyze interactions between dengue virus, virus-specific antibodies, and target cells in PBMC during acute dengue virus infections (quantify and characterize immune complexes, define the major cellular compartments in PBMC supporting dengue viral replication);

5. Assess the utility of plasma sNS1 levels in predicting disease severity for subjects with primary or secondary infection due to any of the four dengue serotypes;

6. Quantitation of viral burden in plasma and cell subsets of PBMCs for all four serotypes in primary and secondary dengue virus infections and determine if there is a correlation between viral load in these compartments and disease severity;

7. Measurement of neutralizing antibody elicited by primary infections, over an extended period of time. Few long-term studies of antibody titer following dengue infection have been performed previously. Neutralizing antibody will be measured on study day 1, 6 months, 1 year, and annually thereafter. Understanding wild type responses will help to set realistic standards for vaccines. Mature secondary responses determined by neutralization six months or more after infection will be correlated with class II HLA type;

8. Determination of memory T-cell responses following primary and secondary dengue infections, over an extended period of time. Understanding wild type responses and the durability of these responses over time will be crucial in setting standards for testing of candidate dengue vaccines;

9. Characterize genetically and functionally the dengue virus-specific T lymphocyte response during, and after dengue virus infections (intracellular cytokine staining, HLA tetramers, T cell receptor gene usage);

10. Analysis of the activation of innate immune responses in vivo during acute dengue virus infections (chemokine gene expression, inhibitory and activating NK receptor expression);

11. Identification of polymorphisms in immune response genes associated with disease manifestations and cellular immune responses during dengue virus infections (MHC class I and II, Fcγ receptor gene, KIR genes, NK receptors) and MHC class I chain-related (MIC) genes (ligands for lectin-like receptors);

12. Continue sequencing portions of the dengue genome from patients with mild dengue fever and those with severe DHF/DSS to test a hypothesis that severity of disease is strain related. In addition, compare the kinetics of plasma viral load and immune responses in primary and secondary infections with different DV serotypes;

d. Methods:

Children were enrolled if they were suspected of having an early DV infection (without evidence of DHF) or a fever without an identifiable source. Inclusion criteria included an oral temperature ≥ 38.5℃, fever onset not longer than 72 hours prior to the initial evaluation, weight > 6kg, age 6 month through 15 years, signed consent by parent or guardian. After informed consent is obtained, subjects are admitted to the hospital and a blood specimen obtained. The result of the plasma test for DV RNA by RT-PCR is available the morning of study day 2. Children who are DV RT-PCR-negative are given the opportunity to leave the study, or to continue in the study for clinical observation. Those children remaining in the hospital undergo inpatient observation until one day following defervescence (fever day +1). Clinical information is collected and recorded daily. Radiographic studies are performed as outlined in the protocol. Serial blood samples are collected and analyzed for routine and dengue-specific blood and plasma tests were conducted to include, but not limited to:

- CBC, WBC differential, AST, Albumin
- Hemagglutination inhibition (HAI) assay for dengue
- Antibody-capture DV IgM/IgG enzyme immunoassay (EIA)
- RT-PCR for dengue, Plasma viremia titers
- Dengue virus isolation in Toxorhynchites splendens and typing
- IL-15, IL-18, MIP-1a, MIP-1b, and MCP-1, CD69, CD38, and Ki-67
- Labeled antibodies to identify T cell subsets, NK cells and B cells
- NS1 (soluble NS1 and anti-NS1 antibodies)
- Complement assays

e. Results:

There were 10 positive PCR cases in this report period (DEN 1 = 5; DEN 2 = 0; DEN 3 = 5, DEN 4 = 0). There were 9 Negative PCR cases. All subjects had ultrasound evaluation for plasma leakage. A subset (n=7) of dengue positive cases had interstitial

fluid sampling done. One subject were withdrawn due to fever over 72 hours. No serious adverse events occurred.

f. Future Plans:

The study is scheduled to stop enrollment on December 2008.Long-term clinical follow-up is ongoing for prior years of enrollment. Analysis for markers that predict disease severity (NS1 protein/antibody levels, immune activation markers), that indicate plasma leakage is or will occur, and that indicate immunity will be done. Statistical analysis of DHF resulting from primary versus secondary DV infections with regard to the role viral serotype, viral burden and virus-antibody complexes plays on resulting disease severity is planned. Characterization of the dengue specific T cell response with regard to the magnitude of T cell expansion during infection and the functional characteristics of these cells is also planned. Measurements of makers of endothelial cell activation are planned. Investigators will submit the amendment protocol request extending the study period for a total of 5 years (31 DEC 2013), 3 years to complete clinical follow up appointments and 2 additional years to complete data analysis.

3. Title of Research Project: A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Infants

a. Background:

Development and U.S. licensure of a tetravalent dengue vaccine for use in U.S. Service Members is a high priority for the U.S. Dept. of Defense. The Kingdom of Thailand seeks licensure of a tetravalent dengue vaccine capable of protecting a broader age range against endemic dengue. The Kingdom of Thailand and the U.S. Army have been cooperatively pursuing these goals through the conduct of basic science and pre-clinical and clinical testing of dengue vaccine candidates. The above named study represents the first clinical trial of an Army dengue vaccine candidate in an overseas (Thailand), infant population.

b. Principal Investigators:

- Robert V. Gibbons, COL, MC (USAMC-AFRIMS)
- Veerachai Watanaveeradej, MD, Phramongkutklao Hospital (PMK),Thailand

Sub Investigators:

- Sriluck Simasathien, MD (PMK)
- Angkool Kerdpanich, MD (PMK)
- Ananda Nisalak, MD (AFRIMS)
- In-Kyu Yoon, MD (AFRIMS)
- Richard G. Jarman, PhD (AFRIMS)
- Supamit Chunsuttiwat,MD., MPH(MOPH)

c. Objectives:

The objective of the study is to assess the safety and immunogenicity of two doses of the WRAIR/GlaxoSmithKline tetravalent dengue vaccine in flavivirus naïve Thai infants. A five year long term safety follow up component was added to the study design. Lastly, investigators explored the safety and immunogenicity (cellular and humoral) of administering a third dose of dengue vaccine.

d. Methods:

Investigators enrolled 51 healthy, flavivirus naïve, Thai infants between the ages of 12 and 15 months. Two doses of the WRAIR/GSK tetravalent dengue vaccine were administered on a 0-6 month schedule. Safety visits were conducted and blood collected at scheduled times to evaluate the development of neutralizing antibodies directed against the four dengue viruses. Long term follow up is being conducted to assess for the occurrence of symptomatic dengue virus infections remote from vaccination. A third dose of dengue vaccine was provided to a subset of the original cohort using a newly derived dengue vaccine candidate. Safety and immunogenicity endpoints are being collected. Clinical study activities will be completed in 2009.

e. Results:

Results from the primary vaccination series (doses #1 and 2) demonstrate the WRAIR/GSK vaccine candidate to be safe and immunogenic. A significant proportion of the cohort develops neutralizing antibodies to all four dengue virus types following two doses of vaccine. Long term follow up reveals no evidence vaccine recipients have an increased incidence of dengue compared to controls in the period remote from vaccination. There were no overt safety signals in the subset of volunteers receiving a third dose of dengue vaccine remote from the primary vaccination series. Final safety and immunogenicity analyses are pending.

f. Future plans:

Clinical activities and final data analysis will be completed in late 2009.

4. Title of Research Project: A Phase I/II, Open, Five-Year, Clinical Follow-Up Study of Thai Children Who Participated in Dengue-003 ("A Phase I/II Trial of A Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Children") with Evaluation of a Booster Dose Given One Year after Primary Dengue Vaccination Series

a. Investigators:

Robert V. Gibbons, LTC, MC, USAMC-AFRIMS
Sriluck Simasathien, MD, Phramongkutklao Hospital (PMK), Bangkok, Thailand

b. Objectives:

The primary objective of this study is to assess the immunogenicity of a booster dose of dengue vaccine administered approximately one year following the second dose.

c. Methods:

1. Enroll seven flavivirus antibody-naïve Thai children who participated in Study Dengue-003 ("A Phase I/II Trial of A Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Children").

2. Provides booster dose of dengue vaccine given one year after the last dose of dengue vaccine in Dengue-003.

3. Assess the persistence of antibody one and two year following the booster dose.

4. Assess the safety and immunogenicity of a booster dose of dengue vaccine administered one year after primary dengue vaccination series.

5. To characterize cell mediated immune responses to each dengue virus serotype two years following the booster dose.

6. Four annual visits follow-up for passive surveillance for hospitalized dengue after 1 month post booster dose.

d. Results:

Seven subjects were enrolled since February 2005 (Year – 1) and all subjects were administering a booster. The protocol amendment 1 was approved and allow for acquire peripheral blood mononuclear cells (PBMCs) and sera to characterize cell-mediated immunity responses to vaccination and correlate these with N antibody titers at year 3 follow-up. The clinical follow-up visit 8 (Year - 4) has been completed since FEB 08. The last follow-up visit will be scheduled in FEB 09 (Year -5). There were no subject withdrawals from the study till date. There is no Sirious Adverse Event (SAE) till date.

Laboratory testing for neutralizing antibodies is complete (below).

Subject Number	Specimen Number	Visit	DEN-1	DEN-2	DEN-3	DEN-4	JE
DEN005-01	D005-006	1	<10	424	13	<10	<10
	D005-021	5	322	>1280	267	>1280	23
	D005-025	6	25	308	25	47	11
	D005-034	7	85	436	58	163	N/A
DEN005-02	D005-005	1	<10	215	<10	<10	<10
	D005-019	5	327	867	>1280	>1280	28
	D005-026	6	32	182	87	344	20
	D005-032	7	25	154	65	302	N/A
DEN005-03	D005-002	1	<10	<10	<10	<10	<10
	D005-008	5	<10	42	12	36	<10
	D005-027	6	<10	<10	<10	<10	<10
	D005-036	7	11	<10	<10	14	N/A
DEN005-04	D005-007	1	<10	<10	<10	<10	<10
	D005-010	5	41	45	381	104	<10
	D005-028	6	<10	17	<10	<10	17
	D005-033	7	36	26	<10	69	N/A
DEN005-05	D005-001	1	15	31	20	260	<10
	D005-020	5	26	46	29	26	10
	D005-029	6	<10	16	13	<10	<10
	D005-035	7	<10	70	18	42	N/A
DEN005-06	D005-003	1	<10	<10	352	62	<10
	D005-009	5	16	37	32	143	<10
	D005-030	6	<10	<10	<10	118	<10
	D005-037	7	41	11	36	262	N/A
DEN005-07	D005-004	1	<10	27	<10	194	<10
	D005-018	5	18	253	17	111	<10
	D005-031	6	<10	65	<10	52	34
	D005-038	7	<10	52	<10	70	N/A

e. Future Plans:

Clinical activities will be end in FEB 09. Investigators will complete testing of samples to fulfill the study objectives and endpoints. Data analysis will continue. The final report will be generated. Scientific manuscripts will be drafted.

5. Title of Research Project: A Phase II, Prospective, Randomized, Double Blind, Placebo Controlled Field Efficacy Trial of a Candidate Hepatitis E Vaccine in Nepal WRAIR# 749, HSRRB Log# A-9117.1

a. Investigators:

Principal Investigators:
- M. P. Shrestha (WARUN)
- R. M. Scott (WARUN)

Associate Investigators:
- S. B. Bajracharya (SBH)
- M. P. Mammen (USAMC-AFRIMS)

- R. A. Kuschner (WRAIR)
- K. S. A. Myint (USAMC-AFRIMS)
- P. R. Pandey (SBH)
- K. J. B. Rana (SBH)
- K. N. Rayamajhi (SBH)
- J. Seriwatana (WRAIR)
- G. R. Shakya (SBH)
- G. B. Thapa (SBH)
- S.K. Shrestha (WARUN)
- N. Thapa (SBH)
- C. Jhang

b. Objectives:

To evaluate the protective efficacy for the prevention of definitive and probable hepatitis E disease provided by the candidate hepatitis E vaccine administered according to a 0, 1 and 6 month schedule.

c. Methods:

A candidate recombinant baculovirus expressed hepatitis E virus (HEV) vaccine was found to be safe and immunogenic in 88 American and 44 Nepalese volunteers. A 20µg formulation was selected for further evaluation in a randomized double blind placebo controlled efficacy trial in susceptible, active duty Nepal Army volunteers. The clinical phase started 30 April 2001 at the Nepalese Army Shree Birendra Hospital. Of 5,571 consenting volunteers screened, 3,692 were susceptible to HEV. Two thousand volunteers (8 females, 1,992 males) were enrolled, receiving either placebo or 20µg of active candidate vaccine. Volunteers were vaccinated at 0, 1, and 6 months with sera collected at months 0, 1, 3, 6, 7, 13, and 24. One tenth of the volunteers were followed on days 1, 3, 5, and 7 after each vaccination for local and general solicited adverse events (SoAE). Non-serious adverse events (NSAE) were recorded for 30 days after each vaccination and serious adverse events (SAE) were also collected throughout the study period. Sera and stool from cases meeting clinical and biochemical criteria compatible with viral hepatitis, were examined for HEV RNA by a reverse transcriptase-polymerase chain reaction, and serologically for HEV IgM and Ig, HAV IgM, HBsAg, HBcIgM and HCV IgG. Pregnancy was also recorded as SAE. Two subjects became pregnant during the study, about one year after third dose. The outcome was favorable in both cases. Seven deaths were documented and verified by Data Safety Monitoring Board (DSMB). They were 4 killed in action, 1 accident, 1 cholangiocarcinoma and 1 undetermined.

The vaccine or placebo doeses received by the 2000 enrolled subjects are as follows:

Number	Dose 1	Dose 2	Dose 3
1794	YES	YES	YES
98	YES	YES	NO
31	YES	NO	YES
77	YES	NO	NO

A total of 1566 subjects returned for concluding visit (Encounter# 8). Clinical study encounter with the subjects was concluded in January 2004.

d. Results:

The clinical phase of the trial has been successfully completed according to the protocol amendment 9. The DSMB unblinded 111 cases of suspected hepatitis following SOP provided by GSK on 23 June 2004. The study results were reported in the HEV Symposium of the American Society of Tropical Medicine and Hygiene (ASTMH) annual meeting in December 2005 as well as in the *New England Journal of Medicine* in March 2007.

The clinical portion of the study was completed in January 2004. A final clinical study report was submitted to the Food and Drug Administration on 21 August 2006.

e. Future Plans:

The WRAIR protocol # 749 reached five year term in October 2006. Memorandum requesting extension of this protocol for 18 months was submitted to Office of Research Management (ORM) on 12 January 2007. The study remains open in order to complete the plan to inform volunteers about the results of the study and the formulation (placebo or vaccine) they received. As of 18 November 2008, letters of appreciation and result of the study to 916 volunteers has been distributed and acknowledgement received. A total of 555 undelivered letters have been returned by Shree Birendra Hospital. Acknowledgement of 200 letters provided to Shree Birendra Hospital for distribution has not been received.

The source document in all subject folders at WARUN and the Regulatory Documents at AFRIMS have been scanned and secured. The electronic files have also been secured off-site. Hard copy source documents and regulatory documents at WARUN has been forwarded to Archivist, Division of Regulated Activities and Compliance U.S. Army Medical Research & Material Command, 1452 Campus Drive, Fort Detrick, MD 21702 and received on 29 September 2009.

6. Title of Research Project: Japanese Encephalitis Surveillance in Nepal

a. Investigators:

- Robert V. Gibbons COL, MC (USAMC-AFRIMS)

- Sanjaya Kr. Shrestha, MBBS, MD (WARUN, Kathmandu)

b. Objectives:

1. To determine JE diagnosis validation in Nepal

2. To determine the percentage of Japanese encephalitis and other causes of encephalitis among blinded samples provided to AFRIMS from Nepal.

c. Methods:

1. Blinded samples provided by National Public Health Laboratory (NPHL) to WARUN without personal identifier.

2. The specimens will then be processed, packaged, and shipped to the Department of Virology, AFRIMS, Bangkok.

3. Definitive quality control testing for JE diagnostics will be done at USAMC-AFRIMS.

d. Results:

In this reporting between 26 October 2007 and 7 Novmber 2008, 1605 blinded clinical encephalitis samplese were provided by National Publich Health Laboratory (NPHL) in Nepal to AFRIMS for JE IgM-capture ELISA. Overall, we detected JE IgM in 12.8 % (205 samples) of the 1603 available samples.

Result of blinded samples tested by JE IgM-capture ELISA assay.

Shipment Date	Specimen	Results			Total
		Positive JE JE_M$\geq$ 40 U	**Borderline** JE_M$\geq$ 30U	**Negative**	
06-Feb-08	SERUM	149	17	768	934
	CSF	33	4	176	213
	No sample				2
	Total	182(15.9%)	21(1.8%)	944 (82.3%)	1147(2)
12-Mar-08	SERUM	0	0	86	86
	CSF	0	0	21	21
	Total	0	0	107(100%)	107
16-Jul-08	SERUM	1	1	258	260
	CSF	0	0	89	89
	Total	1(0.3%)	1(0.3%)	347(99.4%)	349
	TOTAL	**Positive=182+21+2 = 205** **-12.80%**		**1,398** **-87.20%**	**1,603** **(no sample =2)**

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e. Future Plans:

Plans for CY2008 include: continuation of testing of samples provided by NPHL, analysis of the data collected to date; discussion of the data with investigators at WARUN and NPHL; and manuscript writing. Remaining negative samples will be tested for other possible pathogens of encephalitis.

7. Title of Research Project: Influenza Surveillance in Southeast Asia

a. Background:

Influenza is an important cause of morbidity and mortality. Continuous viral surveillance and isolation of influenza viruses provides important information for the creation of annual vaccine formulations based on the identification of new and emerging strains of influenza. AFRIMS has been actively involved in influenza surveillance in Southeast Asia for several years. Expansion of AFRIMS influenza surveillance activities in the region will enhance DoD's ability to detect and respond to an outbreak of pandemic influenza early in the course of the pandemic.

b. Investigators:

- Khin Saw Myint, M.D. (USAMC-AFRIMS)
- Robert V. Gibbons, M.D. MPH (USAMC-AFRIMS)
- Richard G. Jarman,Ph.D (USAMC-AFRIMS)
- Kumnuan Ungchusak, M.D., MPH (MOPH, Thailand)
- Darunee Tannitisupawong, M.D. (USAMC-AFRIMS)
- John Mark Velasco, M.D. (USAMC-AFRIMS)
- Sanjaya K. Shrestha, M.D. (WARUN, Nepal)
- Tandin Dorji, MSc (PHL, Bhutan)
- Julie Pavlin, M.D. (GEIS, Thailand)

c. Objectives:

(a) To collect and characterize influenza viruses circulating within the human population in Asia including Thailand, Nepal, the Philippines, Bhutan and from the US Embassy/Consulate in the region.

(b) To provide influenza surveillance data to the US CDC and WHO surveillance network towards the annual re-formulation of the influenza vaccine.

(c) To report the circulating influenza strains and other respiratory pathogens to the Ministry of Health of host countries.

d. Methods:

Samples were collected from patients with clinically suspected influenza infection (case definition includes fever or history of fever $\geq 38°$C within 72 hours with cough or sore throat). Participating physicians and staff identified patients who met the case definition during clinic visits. Emphasis was placed on quality samples that may provide genetic data for future influenza vaccines rather. Clinical history forms, including basic demographic and clinical information, were completed by the OPD nurse or AFRIMS staff. Nasal specimens were collected and tested at field sites for rapid feed-back to the physicians and patients. Nasal/throat swabs were collected and placed in viral media and stored at -70°C. All specimens were shipped on dry ice to AFRIMS for typing and subtyping using molecular techniques. An aliquot was shipped to USAFSAM, San Antonio, Texas, for virus isolation and other definitive diagnostic tests.

e. Results:

AFRIMS works in close collaboration with the US and Thai CDCs, the Thai Ministry of Public Health, public health officials in Nepal, Bhutan and the Philippines, and with NAMRU-2. The influenza surveillance is divided into individual country projects each for Thailand, Nepal, U.S. Embassies in the region, Bhutan and the Philippines. Provision of staff, equipment, infrastructure development, and training is well underway at all sites. AFRIMS has already set up training on molecular diagnostics in Kathmandu, Kamphaeng Phet, and in the Philippines. The infrastructure of a dedicated respiratory pathogens laboratory is already completed in these sites. This will allow immediate processing of influenza samples, and ensure on-time reporting. Construction of a BSL-3 laboratory is well underway at AFRIMS. The respiratory laboratory will be equipped with a real-time PCR machine, serology set up, viral isolation, computers for data entry, and capabilities for specimen storage and archiving.

Progress on influenza surveillance in U.S. citizens at U.S. Embassies/Consulates in Asia: subject enrollment started since the protocol was approved by HURC on Feb 2006. There are now a total of 14 medical units/clinics from 13 countries in Asia participating in this study - Thailand (Bangkok), Burma (Rangoon), Bangladesh (Dhaka), India (New Dehli), Pakistan (Islamabad), Mongolia, Laos (Vientiane), Malaysia (Kuala Lumpur), Sri Lanka (Colombo), Vietnam (Hanoi and Ho Chi Minh City), Nepal (Kathmandu), China (Beijing), and Philippines (Manila). Total number of subjects enrolled since the study started until Dec 08 is 129; majority was from the US Embassy Medical Unit in Bangkok. Rapid diagnostic tests performed on site showed the following: 18 (13.9%) FLU A, 4 (3.1%) FLU B and 5 (3.8%) FLU A+B. Real-Time PCR for Influenza A/B performed at AFRIMS showed the following: 9 (6.9%) FLU A/H1, 27 (20.9%) FLU A/H3 and 15 (11.6%) FLU B. Virus isolation results were available on 96 specimens: 16 (12.4%) Influenza A, 8 (6.2%) Influenza B, 1 (0.7%) Parainfluenza 2, 2 (1.5%) Parainfluenza 3, 1 (0.7%) herpes simplex virus, and no respiratory virus was isolated in 68 (52.7%).

Progress on influenza surveillance in Nepal: During the period from initiation of the study until 30 November 2008, 918 specimens were received from active sentinel surveillance sites and outbreaks in Nepal. Rapid diagnostic test showed 206 (22.5%) FLU A and 50 (5.5%) FLU B out of 917 specimens. Real-time PCR done at AFRIMS reported 296 (32.6%) as FLU A/H1, 59 (6.5%) FLU A/H3, 1 (0.1%) (1) FLU A/H3 + FLUB, 3 (0.3%) FLUA/Un-subtype and 96 (10.6%) FLU B out of 907 specimens. Viral isolation performed at USAFSAM showed 261 (32.2 %) FLU A, 68 (8.4 %) FLU B, 4 adenovirus (0.5%), 2 Coxsackie B (0.2%), 1 Echo virus (0.1%), 4 Enterovirus (0.5%), 5 Parainfluenza-1 (0.6%), 2 Prainfluenza -2 (0.2%), 4 Parainfluenza-3 (0.5%), 1 Mumps virus (0.1%) and 4 Herpes Simplex Virus (0.5%) out of 810 specimens. No A/H5N1 has been isolated to date. Real time PCR assays for the detection of influenza A & B viruses and their subtypes have been initiated at AFRIMS's lab in Nepal (WARUN).

Progress on influenza surveillance in the Philippines: A total of 569 influenza-like illness (ILI) cases were investigated from the following sites in the Philippines: 1) 5 barangay health center (BHC) sentinel sites, Cebu City 2) Armed Forces of the Philippines Medical Center (AFPMC), Quezon City, and 3) U.S. Embassy Medical Unit, Manila. Male to female ratio of the ILI cases was 1.1 and with age range of 6 months to 63 years old. Majority of the ILI cases were from the 6 months to 4 years old age group and 5 to 9 years old age group at 63% and 23%, respectively. Influenza real time PCR was performed on 425 specimens with 132 (32%) positive for influenza. Breakdown of the influenza PCR results are as follows: 65 (15%) Influenza A/H3, 50 (12%) Influenza B, 15 (4%) Influenza A/H1, 4 (1%) Influenza A (un-subtyped), and 290 (68%) were negative. One specimen was positive for both Influenza A/H3 and Influenza B. Virus isolation results were available for 332 of the specimens: 50 (15%) Influenza A, 28 (8%) Influenza B, 14 (4%) Parainfluenza 1, 13 (4%) Parainfluenza 3, 13 (4%) Adenovirus, 6 (2%) Coxsackie B, 3 (1%) Parainfluenza 2, 2 (1%) Enterovirus, and no respiratory virus was isolated in 199 (60%) of the specimens. Single samples of each of the following viruses were also isolated: Echovirus, Herpes Simplex Virus, Respiratory Syncitial Virus, and a specimen with concurrent infection of Influenza A and B.

Progress on influenza surveillance in Thailand: Total number of subjects enrolled from Kamphaeng Phet and Sangkhlaburi since the study started in April 2007 until temporary halt in March 2008 was 604. Oseltamivir (O) resistance was assessed at the US CDC on four H1N1 viruses isolated from specimens collected in Nov 2007 in Kamphaeng Phet. One of the four H1 strains was found to be O-resistant.

A total of 151 cases that fit the ILI criteria were collected from an ILI outbreak in Sangkhlaburi (Jan-Feb 2008). Nasal swabs were taken to test with QuickVue and throat swabs for the diagnostic panel (viral isolation, RT-PCR and characterization). The outbreak was determined to be FLUB-Malaysia-like. Thirty-seven percent were positive for FLU B by the rapid QuickVue test (done on site). Eighty-eight (58%) were positive for Flu B by RT-PCR, 2 (1%) FluA/H3 by RT-PCR, 75 (50%) were isolation positive for flu B and were characterized as B-Malaysia-like by molecular sequencing. Five of these were sent to the US CDC for HAI testing against B-Malaysia antiserum. Isolation also revealed the following from the outbreak specimens: 2 Adenovirus, 1 Coxsackie B, 2 Echo Virus, 1 Enterovirus, 1 HSV, 1 Parainfluenza 1, 52 (34%) unknown.

Progress on influenza surveillance in Bhutan: AFRIMS has established a relationship with Bhutan Public Health Laboratory and Bhutan Ministry of Health to enhance the country's capabilities to perform influenza and other emerging pathogen surveillance by significantly increasing laboratory and information technology infrastructure and through the provision of training. Influenza-like illness specimens are collected from Thimphu, Punakha and Paro with expansion to Phuentsholing, Gelephu, and Mongar in later phases of the study. Thimphu is the capital city of Bhutan with an estimated population of 80,000, the highest in the country. Paro and Punakha are two districts frequented by tourists and are located close to Thimphu. Three hospitals (Jigme Dorji Wanghck National Referral Hospital, Thimphu; Punakha Hospital; Paro Hospital) are initially involved in the study.

Twenty five ILI service specimens were collected from Bhutan from Oct-Dec 2008. Influenza real-time PCR subtyping done at AFRIMS showed the following results: 15 (60%) positive for influenza A (H3), and 2 (8%) positive for influenza B. No respiratory virus was detected in 8 (32%) of the specimens tested.

f. Future Plans:

1. To expand surveillance sites in Nepal, Bhutan and the Philippines.

2. To expand surveillance to other countries in the region.

3. Set up BSL-3 facility at AFRIMS.

4. Set up Real-Time RT-PCR in Bhutan.

8. Title of Research Project: Phase II, randomized, double-blind, single center, controlled study of two doses of different formulations of the WRAIR live attenuated tetravalent dengue vaccine compared to a placebo control, administered on a 0-6-month schedule, to healthy adults

a. Investigators:

Principal Investigators:
- Robert V. Gibbons, LTC, MC (USAMC-AFRIMS)
- Veerachai Watanaveeradej, MD, Phramongkutklao Hospital (PMK),Thailand

Sub Investigators:
- Sriluck Simasathien, MD (PMK)
- Danaband Phiboonbanakit, MD (PMK)
- Angkool Kerdpanich, MD (PMK)
- Adisorn Lumpaopong,MD (PMK)
- Atik Sangahsapaviliyah, MD (PMK)
- Nopaorn Phavichitr, MD (PMK)
- Ananda Nisalak, MD (AFRIMS)

- Stephen J. Thomas, MAJ(P), MC (WRAIR)
- Kenneth H. Eckels, PhD (WRAIR)
- J. Robert Putnak, PhD (WRAIR)
- In-Kyu Yoon, MD (AFRIMS)
- Richard G. Jarman, PhD (AFRIMS)

b. Objectives:

1. To evaluate the safety of T-DEN vaccine in terms of the occurrence of solicited adverse events (AE) within the 21-day solicited follow-up period following administration of study vaccine dose 1.

2. To explore the immunogenicity of T-DEN vaccine in terms of the GMT of neutralizing (N) antibody to each DEN serotype (DEN-1, -2, -3 and -4) determined 30 and 90 days after vaccine dose 2.

c. Methods:

1. Screen and enroll 120 Thai subjects, 20-25 year old healthy males and non-pregnant healthy females.

2. Randomized, double-blind, placebo-controlled study with 3 groups: T-DEN (post-transfection) F17, T-DEN (post-transfection) F19 and Placebo control.

3. Provide 2 doses of the tetravalent dengue vaccine or placebo at 0 - 6 month apart.

4. Evaluate the safety of vaccine in terms of AE, SAE, alert values, abnormal DEN physical examination finding and suspect or confirmed dengue.

5. Evaluate the immunogenicity of vaccine in terms of N antibody and measurable dengue viremia

6. One screening visit and 10 study visits

7. The intended duration of the study will be 9 months (in addition to the screening period).

d. Results:

A total of 120 volunteers were enrolled and randomized to receive one of two dengue vaccine formulations or placebo in April 2007. One hundred sixteen subjects received dose 2 and complete study visit. There are 4 subjects lost to follow up (3 subjects after 1st dose and 1 subject after 2nd dose). The final study visit occurred 14 February 2008.

There are 3 SAEs reported (1 abdominal hernia, 1 ectopic pregnancy and 1 appendicitis) none of which were demmed related to study vaccine. There are 2 pregnancy cases reported. There are 2 pregnancy reported during this report period.

There were no volunteers which met clinical or laboratory criteria for suspected dengue and no Alert LaB Value was found.

Laboratory testing for JE PRNT was done at AFRIMS in NOV 08.

An interim safety analysis performed on cleaned data collected for 31 days after the first study vaccine dose (days 0-30 post dose 1) in the entire cohort (2 vaccine and 1 placebo group). The summary results showed the vaccine was well tolerated in a highly primed adult population.

e. Future Plans:

Lab testing for Q-PCR will be done at AFRIMS in JAN 09. The specimens will be sent to test at location as specified in the protocol in JAN 09. The assays supporting immunogenicity objectives and endpoints will be complete. Completion of the data analysis and filing of the final study report is expected to occur in CY2009.

9. Title of Research Project: Use of Geographic Information System (GIS) to Establish a Community-Based Biosurveillance Infrastructure in Cebu City, Philippines (WRAIR #1385)

a. Investigators:

Principal Investigators:

- In-Kyu Yoon, M.D. (USAMC-AFRIMS)
- John Mark S. Velasco, M.D. (PAVRU)

Associate Investigators:
- Charity Ann Butac-Cardenas, M.D. (PAVRU)

b. Objectives:

1. To enumerate members of a geographically defined population

2. To establish baseline demographic, geographical, environmental and epidemiological data of Guadalupe and Banawa households using Geographic Information System (GIS) and Global Positioning System (GPS) technology

3. To facilitate future determination of relationships between selected disease transmission and environmental factors (health facilities, high population density, social and economic conditions, etc.) through spatial analytical techniques

c. Methods:

Demographic and health questionnaires were administered by pre-trained interviewers to consenting non-transient adult members representing households located in the communities of Guadalupe and Banawa, Cebu City, Philippines. Existing high-resolution orthorectified photos of Guadalupe and Banawa from the Cebu City GIS Center were used to facilitate generation of polygon-shape data files representing the physical location of the houses where the households resided. Unique household ID numbers were assigned to each polygon as well as to individual household members. Collected information included: location of the household; name, age, and sex of each household member; intended duration of residence in the study area; level of education of household members; health facility utilization; other items such as construction material of the house, ownership of telephones etc. For the purposes of this study, a household was defined as a group of people who share living accommodations and meals (i.e. "same cooking pot"). Data gathered was encoded into a custom made SQL database with the data per household linked to the polygon-shape data files in Arcview. Random manual verification of the encoded data in the database as compared to the source documents was performed by study staff. House-to-house verification was also done to determine accuracy of the encoded data in the GIS database with the corresponding household ID and associated polygon-shape file data representing the physical structure.

d. Results:

As of December 2008, a total of 9,985 households in Guadalupe and Banawa have been interviewed representing 45,151 individuals. This corresponds to a population coverage of approximately 94% of the individuals (as compared to the 2007 population census). Average household size was calculated at 4 members per household. Male to female ratio was reported as 0.94 with majority of the population reported as either single or legally married at 26,004/ 45,151 (56%) and 14,663/ 45,151 (32%), respectively. Majority of the population belonged to the 20-29 and 30-39 years old age groups at 21% and 14%, respectively. Projected health facility utilization data of adults and children for a hypothetical fever was elicited from 9,896 households while actual health facility utilization during the past year for a febrile illness was determined from 6,776 households. Out of 9,896 households, 6,422 (65%) indicated that a member of their household had a fever during the past year. The total number of individual household members who reported having had a fever during the past year was 11,679 (26%).

Out of 9,852 households who answered the question whether they plan to transfer or move out of their present residence in the near future, only 211 (2%) answered in the affirmative while 2,129 (22%) were unsure or answered "don't know." Mobile phone

ownership by any member of the household was significantly higher with 8,078/ 9,884 (82%) households versus telephone landline ownership which was reported at 3,549/ 9,884 (36%). Ownership of pets or animals was reported among 3,945/ 9,891 (40%) households.

e. Future Plans:

Addition of other data layers from various publicly available data sources such as those collected by local health centers and hospitals (i.e., Field Health Service Information System (FHSIS) and Philippine Integrated Disease Surveillance and Response (PIDSR)), local AFRIMS projects, as well as other collaborative research projects will be done once the database has been finalized and appropriate checks have been instituted. This database will be used to estimate and create models of spatial and temporal disease spread, transmission, and clustering as applied to other succeeding projects.

10. Title of Research Project: Sentinel Surveillance for Emerging Diseases Causing Hospitalized Dengue-like Illness in Cebu, Philippines (SEDC)

a. Principal Investigators:

Armed Forces Research Institute of Medical Science (AFRIMS):
Department of Virology

- Maria Theresa Alera, M.D (Vicente Sotto Memorial Medical Center, Cebu City, Philippines)
- Charity Ann Y. Butac, M.D. (Philippines AFRIMS Virology Research Unit, Cebu City, Philippines)

Other Study Personnel:

Armed Forces Research Institute of Medical Science (AFRIMS):
Department of Virology

- In-Kyu Yoon, M.D
- Thidarat Intararit, R.N.

Vicente Sotto Memorial Medical Center

- Allan Monteclar, M.D.

Philippines AFRIMS Virology Research Unit

- John Mark Velasco, M.D.

b. Objectives:

Primary objectives

To determine the proportion of hospitalized dengue-like illness that is caused by dengue viruses.

Secondary objectives

1. To determine the proportion of hospitalized dengue-like illness that may be caused by:

> a. Leptospirosis
> b. Chikungunya
> c. Scrub typhus
> d. Murine typhus

2. To determine the seroepidemiologic profile of patients who present with dengue-like illness

3. To determine association between dengue serotype and the following variables:

c. Methods:

Study Design:

This was a cross-sectional, hospital-based passive surveillance study conducted among admitted patients in Vicente Sotto Memorial Medical Center (VSMMC), a tertiary government hospital located in Manila, Philippines

Study Population:

The study population consisted of male and female patients 2 years old and above who presented with dengue-like syndrome and with a signed informed consent. Patients with DLS were enrolled in the study if they met the following inclusion criteria: any patient with history of fever (temperature of $38^{o}C$ and above) within the past 7 days with either one of the following criteria: one of the following: hemorrhagic manifestations (positive tourniquet test, petechiae, ecchymoses, mucosal bleeding or bleeding from other sites) or two of the following: headache, generalized rash, myalgias, arthralgias, retro-orbital pain.

Data Collection:

Patients who met the enrollment criteria were interviewed and examined. The patient's personal, demographic, clinical and laboratory data were recorded. Acute blood specimens were drawn. Initial laboratory testing focused on probable etiologies given the

recognized endemic pathogens. Cases that remain undiagnosed after the initial stage of testing were further characterized using specialized testing to identify emerging pathogens. Patients were advised to come back 14 days after onset of illness where a convalescent serum was drawn.

Laboratory testing will be conducted in Stages:

Stage 1 – Includes clinical laboratory tests routinely ordered by the Vicente Sotto Memorial Medical Center attending physician as part of the management of the cases based on the prevailing standards of care of the various conditions in the Philippine setting.

Stage 2 – AFRIMS In-house anti Dengue IgM/IgG EIA will be performed to help rule in/rule out dengue etiology. Dengue virus RT-PCR/Nested PCR will be performed to determine the serotype of dengue positive acute specimens. Specimen with negative/inconclusive results will be subjected to the next level of testing.

Stage 3 – Refers to laboratory tests to screen for other selected etiologies of dengue-like illness. This testing will be done utilizing Leptospirosis IgM EIA, Chikungunya IgM EIA and Scrub/Murine Typhus EIA. Other laboratories may be enlisted to perform specialized pathogen diagnostic testing as needed.

  d. Results:

Dengue-like Syndrome

    From January to December 2008, 176 patients were enrolled into the study. There were 14 lost to follow-up and 2 withdrawals. No Serious Adverse Events (SAEs) were reported. AFRIMS JE/ dengue EIA and/ or dengue RT-PCR data was available on 135 of the subjects. One hundred nineteen (88%) out of the135 subjects were laboratory confirmed dengue infections. Demographics of the laboratory confirmed dengue cases are as follows: age range of 2 to 32 years old with majority (96%) with age less than 15 years old; male to female ratio was 1.25. One hundred seven (90%) subjects were diagnosed as acute secondary infections and only 4 (3%) cases were diagnosed as acute primary infections. The remaining cases were either suggestive of secondary dengue infection (6%) or it unable to be differentiated whether it was a secondary or primary dengue infection (1%). Specific breakdown of the clinical diagnosis of the laboratory confirmed dengue cases as follows: 53 (45%) DHF gr 2, 31 (26%) DHF gr 1, 29 (24%) DSS (DHF gr 3/gr 4), 4 (3%) DF, and 2 (2%) systemic viral infection. Serotype data was available on 68 subjects with all 4 serotypes documented to be circulating with DEN-3 (82%) predominating. Confirmatory laboratory testing is still being done for the 14 non-dengue cases.

e. Future Plans:

Subject recruitment and clinical follow-up will continue as outlined in the approved protocol. The demographics/ base line data will be analyzed for those enrolled during CY 2008.

11. Title of Research Project: Prospective Studies of Avian Influenza Transmission in Cambodia and Thailand

a. Background:

In Asia, response to the H5N1 avian influenza crisis has involved major changes in live bird markets, the culling of millions of domestic birds, the experimental administration of avian influenza H5N1 vaccines to domestic poultry, restrictions on importation and exportation of poultry, and increased biosecurity measures in poultry production facilities. While these interventions have reduced or eliminated transmission in some geographical regions, avian influenza virus has continued to spread. Some have argued that since the virus is prevalent among Asia's wild duck species and causes little disease in ducks, H5N1 eradication may be nearly impossible.

Public health professionals and political officials have been making pandemic preparations. Response plans are based upon the best available disease modeling information. Influenza modeling is complex and requires many assumptions regarding virus transmission, infectivity, proportion of susceptibles, success of interventions, etc. Currently, because H5N1 epidemiological data are sparse, the models that have guided H5N1 containment policy have been based upon data gathered from other influenza strain transmission studies. These assumptions may or may not be valid for avian influenza transmission.

The need for good epidemiological data is among the highest research priorities regarding the possible H5N1 pandemic. As human disease detection in Asia is based upon clinical encounters, little is known regarding subclinical infections or risk factors for H5N1 infection. The currently proposed study intends to address such needs through the evaluation of two cohorts in two nations that have experienced H5N1 transmission, Thailand and Cambodia. Our *long term goal* in this proposed research is to characterize epidemiologically clinical and subclinical H5N1 transmission such that public health officials may design appropriate pandemic influenza surveillance and control measures.

b. Investigators:

Principal Investigators:
- Gregory Gray, MD, MPH (University of Iowa, USA)
- Pathom Sawanpanyalert, MD, DRPH (Ministry of Public Health, Thailand)
- Benjawan Khuntirat, PhD (USAMC-AFRIMS)

Co-Investigators:
- Robert V. Gibbons, LTC, MD, MC (USAMC-AFRIMS)
- Kumnuan Ungchusak, MD (Ministry of Public Health, Thailand)
- Krongkaew Supawat, MS (Ministry of Public Health, Thailand)
- In-Kyu Yoon, LTC, MD, MC (USAMC-AFRIMS)
- Richard G. Jarman, MAJ, PhD, MC (USAMC-AFRIMS)
- Khin S. Myint, MD (USAMC-AFRIMS)
- Darunee Tannitisupawong, (USAMC-AFRIMS)
- Patrick Blair, LCDR, PhD (NAMRU-2)
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- Thomas F. Wierzba, PhD (NAMRU-2/NIPH, Cambodia)
- Ung Sam An, MD, MPH (National Institute of Public Health, Cambodia)
- Chap Seak Chhay, MD, MPH (National Institute of Public Health, Cambodia)
- Whitney Baker, MPH (University of Iowa, USA)
- Jeff Dawson, ScD (University of Iowa, USA)
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- Rungrueng Kitphati, MD (Ministry of Public Health, Thailand)
- Wattana Auwanit, PhD (Ministry of Public Health, Thailand)
- Siriphan Saengaroon, MS (Ministry of Public Health, Thailand)

c. **Objectives:**

1. To prospectively monitor a cohort of human populations with poultry contact for evidence of avian influenza A infection.

2. To determine risk factors for H5 influenza A infection among human populations with close domestic poultry exposure.

3. To characterize H5 influenza isolates associated with human infection through cDNA sequence studies.

d. **Methods:**

1. Study Site Selection: In both study countries (Cambodia & Thailand), we identified geographical sites where previous H5N1 transmission has been reported, detected, or suspected and invite study subjects to enroll. The selected study sites are located in Kampong Cham, Cambodia and Kamphaeng Phet, Thailand. Eight field sites were established within each study site.

2. Study Recruitment: Field teams actively recruited 800 volunteers (100 volunteers per field site) among persons working or residing in each study site.

Systematic sampling was used to identify 100 households for enrollment. Within each household, the investigators randomly selected an adult for enrollment.

3. Specimen collection: A blood sample (10 ml) will be collected from each subject at enrollment and at each annual follow-up visit for three consecutive years. If the subject develops flu-like symptoms, a blood sample (10 ml) and respiratory specimens (nasal and throat swabs) will be collected. At day 60 after the ILI investigation, a convalescent blood specimen will be collected from the cohort subject.

4. Follow-up: Throughout the three year study, a weekly follow-up is conducted to active surveillance for influenza-like illness (ILI) among cohort study participants. Regular annual follow-up is also performed.

5. Investigating influenza-like illness: Study staff conducts a home visit when a cohort subject develops signs and symptoms of an influenza-like illness. If the subject meets the ILI case definition as outlined in the study protocol, blood and respiratory specimens will be collected. RT-PCR of respiratory specimens is performed to evaluate if the subject is infected with influenza A or B.

6. Family study of influenza transmission: Should RT-PCR from respiratory specimens reveal the subject is influenza A positive, study staff again visits the home and invite family members of the subject's to participate in a case contact study of influenza transmission. A blood specimen (10 ml) is collected from each participating family member. Respiratory (nasal and throat swabs) specimens are additionally collected from each contact subjects with ILI symptoms. RT-PCR of respiratory specimens is performed to evaluate if the contact subject is infected with influenza A or B. A weekly follow-up is also conducted among the contact subjects for 60 days. At day 60, a convalescent blood specimen will be collected from each contact subject and the weekly follow-up is completed.

7. Investigating a confirmed high pathogenicity avian influenza (HPAI) virus infection and a suspected avian influenza virus infection: Should a subject have a <u>POSITIVE RT-PCR for H5N1</u>, PI will notify the designated Ministry of Public Health representative and co-investigators. Further investigation will be conducted per MOPH's instruction. In addition, if a suspected avian influenza virus infection is detected, the MOPH will be immediately notified. Further investigation will be done according to MOPH's guideline.

8. Laboratory testing:

- Molecular study using RT-PCR from respiratory specimens is immediately conducted at KAVRU to evaluate if the subject is infected with influenza A or B.

- Serological studies for influenza A are subsequently performed at MOPH and University of Iowa laboratories using hemagglutination inhibition and microneutralization tests.

- Virus culture and identification of the influenza A positive samples are subsequently conducted at MOPH laboratory to validate the molecular and serological assays, as well as to provide isolates that might be molecularly studied with sequencing for evidence of genetic drift.

e. Results:

In Thailand, 800 cohort subjects were enrolled in the three year surveillance study of avian influenza transmission. The enrollment started in April 2008 and completed in October 2008. As of 31 December 2008, four cohort subjects were withdrawn from the study. Three subjects were withdrawn due to death and one subject was withdrawn due to inability to comply with the study protocol.

Enrolled Cohort Subjects

Age (Years)	Male	Female	Total (%)
20-29	18	22	40 (5)
30-39	57	91	148 (18.5)
40-49	91	141	232 (29)
50-59	85	111	196 (24.5)
60-69	52	62	114 (14.3)
70-79	29	32	61 (7.6)
≥ 80	7	2	9 (1.1)
Total (%)	339 (42.4)	461 (57.6)	800 (100)

During the year 2008, 26 ILI investigations were conducted. Sixteen ILI investigations were found to be positive for influenza virus by RT-PCR testing; the remaining ten were negative for influenza virus. Twelve of 16 ILI cases were positive for influenza A (H1=7, H3=5), three were positive for influenza B, and one was positive for influenza A (H3) and B. Thirteen family transmission studies were conducted; 30 case contact subjects were enrolled. It was later found out that two case contact subjects did not meet the enrollment criteria (did not live under the same roof as the cohort index subjects). These two case contact subjects were subsequently withdrawn from the study. Of the remaining 28 case contact subjects, four had ILI symptoms and all were positive for influenza A virus (H1=3, H3=1) with the same subtype as the respective index cohort subjects.

Enrolled Case Contact Subjects

Age (Years)	Male	Female	Total (%)
1-19	6	7	13 (43.3)
20-29	2	1	3 (10)
30-39	2	3	5 (16.7)
40-49	3	1	4 (13.3)
50-59	1	1	2 (6.7)
60-69	1	0	1 (3.3)
70-79	1	1	2 (6.7)
≥ 80	0	0	0 (0)
Total (%)	16 (53.3)	14 (46.7)	30 (100)

f. Future Plans:

Weekly and annual follow-ups, ILI investigation, and Family Study of Influenza Transmission of the 796 cohort subjects will be continued.

F. Department of Retrovirology, AFRIMS FY08 Research Accomplishments

1. Title of Research Project: A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp120 B/E (AIDSVAX® B/E) Boosting in HIV-uninfected Thai Adults (RV144, HSRRB Log No. A-11048, BB-IND 8795)

a. Investigators:

- Dr. Supachai Rerks-Ngarm and Dr. Supamit Chunsutthiwat
  Department of Disease Control,
  Ministry of Public Health
  Nonthaburi, Thailand

- COL Sorachai Nitayaphan
  RTA Component, AFRIMS
  Bangkok, Thailand

- Prof. Punnee Pitisuttithum and Assoc. Prof. Jaranit Kaewkungwal
  Faculty of Tropical Medicine, Mahidol University
  Bangkok, Thailand

b. Objectives:

Primary: To determine whether immunizations with an integrated combination of ALVAC-HIV (vCP1521) boosted by AIDSVAX® gp120 B/E prevent HIV infection in healthy Thai volunteers. Secondary: To determine whether immunization with this

vaccine combination results in reduced HIV viral load "set point" among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To determine whether immunization with this vaccine combination results in an increased CD4 count measured at viral load "set point" among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To confirm the safety of this vaccine combination in Thai volunteers. To evaluate whether participation in this HIV vaccine trial is associated with behavior change that may increase the risk of HIV infection.

c. Methods:

This will be a community-based, randomized, multicenter, double-blind, placebo-controlled clinical trial (vaccine: placebo = 1:1). Screening of potential volunteers will be carried out under a separate protocol entitled "Screening and evaluation of potential volunteers for a trial in Thailand of a candidate preventive HIV vaccine" (RV148). Eligible volunteers will be enrolled over approximately one year. The statistical assumptions of the study will require that 16,000 persons enroll into the study. Vaccinations for each individual will occur over a 24-week period (0, 4, 12, 24 weeks). Women will be tested for pregnancy and pregnant volunteers will not be vaccinated. The volunteers will be followed with HIV testing every 6 months for 3 years after immunization. Blood will be collected for plasma (for diagnostics and HIV-specific antibodies) at 0, 24 and 26 weeks, and every 6 months during the follow-up phase. The blood collection at 0 and 52 weeks will also be used for cryopreservation and archiving of PBMCs (for HIV-specific cellular immune responses). At week 24 and at each six-month follow-up visit, volunteers will have HIV testing, preceded by pretest counseling and followed (approximately 2-3 weeks later) by post-test counseling. Assessment of HIV risk behavior will be performed at baseline and at each 6-month follow-up visit. Education on risk behavior reduction will be given at each vaccination visit and at each post-test counseling visit.

d. Results:

The first volunteer injected on 20 October 2003. Enrollment ceased 31 Dec 2005. 16,402 persons were enrolled and 16,396 received at least one vaccination. 13,974 persons received 4 vaccinations (85.2%). Follow up of volunteers will be completed 30 June 2009.

The Data Monitoring and Safety Board met in July 2008 met for the last time and recommended the trial continue until completion.

e. Future Plans:

The final analysis of trial data is being planned and expected release of results will occur in Q3 FY2009.

2. Title of Research Project: Extended Evaluation of the Virologic, Immunologic, and Clinical Course of Volunteers Who Become HIV-1 Infected During Participation in a Phase III Vaccine Trial of ALVAC-HIV and AIDSVAX® B/E (RV152, WRAIR #1184)

a. Investigators:

- Dr. Supachai Rerks-Ngarm and Dr. Supamit Chunsutthiwat
  Department of Disease Control
  Ministry of Public Health
  Nonthaburi, Thailand

- COL Sorachai Nitayaphan
  RTA Component, AFRIMS
  Bangkok, Thailand

- LTC Robert Paris , COL Jerome Kim and Dr. Mark de Souza
  Department of Retrovirology,
  U.S. Component, AFRIMS
  Bangkok, Thailand

- Assoc. Prof. Jaranit Kaewkungwal
  Faculty of Tropical Mediciine, Mahidol University
  Bangkok, Thailand

b. Objectives:

This protocol seeks to establish whether a vaccine effect on HIV-1 viral load results in a reduction in the number of composite HIV-related clinical endpoints, which also includes a biomarker (CD4 count) component. This study also includes assays of both cellular (e.g., intracellular cytokine staining, CTL) and humoral (neutralizing antibody) responses to identify putative correlates of vaccine-associated immunity, as well as virologic characterization of infecting viruses by genotyping and selective sequencing to assess for selective vaccine efficacy.

c. Methods:

Volunteers attend study visits every 3 months to receive a clinical assessment, CD4 count, and viral load measurement, as well as collection of PBMC's and plasma for research assays.

d. Results:

The protocol began enrollment in May 2006 and is on-going as HIV-infected volunteers from the phase III trial are identified Study results will be analyzed when approximately 80 primary composite endpoints have accumulated.

e. Future Plans:

The protocol will continue to enroll HIV-infected participants from RV144 until the scheduled end of that protocol in June 2009. Volunteers will be followed for 5 or more years from enrollment.

3. Title of Research Project: A Phase I Double-Blind Randomized Dose Escalating, Placebo-Controlled, Study of Safety and Immunogenicity of WRAIR/NIH Live Recombinant MVA-CMDR (HIV-1 CM235 env/ CM240 gag/pol) Administered by Intramuscular (IM) or Intradermal (ID) Route In HIV-Uninfected Adults (RV158, WRAIR #1143)

a. Investigators:

- Dr. Mary A. Marovich
  U.S. Military HIV Research Program
  Rockville, MD, USA

- Professor Prasert Thongcharoen
  Dept of Microbiology, Faculty of Medicine, Siriraj Hospital
  Mahidol University
  Bangkok, Thailand

- Dr. Somchai Sriplienchan, LTC Robert Paris, Dr. Mark de Souza,
  COL Jerome Kim
  Department of Retrovirology
  U.S. Component, AFRIMS
  Bangkok, Thailand

b. Objectives:

Primary: To evaluate the safety and tolerability of MVA-CMDR (HIV-1 CM235 ENV/CM240 GAG/POL) administered by IM or ID injection to HIV-uninfected adult volunteers.

Secondary: To evaluate the ability of MVA-CMDR (HIV-1 CM235 ENV/CM240 GAG/POL) to induce HIV antigen specific cellular and humoral immune responses.

c. Methods:

This is a phase I double-blind, randomized, dose-escalating, placebo-controlled, study. Healthy HIV-uninfected adult volunteers (18 to 40 years old at the time of enrollment) will be enrolled up to 90 days prior to the first vaccination. Volunteers will be randomized to vaccine or placebo in a 5:1 ratio. Vaccinations will be on Days 0, 28, and 84 of each volunteer's schedule; each vaccination will be followed with a phone call (within 24 to 48 hours) and a safety visit (within 14 days); and each subject will be

followed up for approximately 40 weeks after the third vaccination. Total study duration will be 52 weeks for each volunteer. Volunteers attend study visits as scheduled to receive a clinical assessment, routine hematology, serum chemistry, liver function tests, urinalysis, ECG, and related laboratory tests for safety monitoring during the study conduct.

d. Results:

The protocol began enrollment on 27th November 2007 in Thailand. Twelve volunteers were enrolled at two sites (6 each): Siriraj Hospital and the AFRIMS Clinical Trial Center at Bumrungrad. All volunteers received their first vaccination in January 2008 and completed follow up in October 2008. Preliminary unblinded safety data at all three sites (two in Thailand, one in US) show the vaccine to have an excellent safety profile, with the majority of reactogenicity being due to local cutaneous reactions in the group administered vaccine by the ID route (Grade 1 erythema, induration, pain, and tenderness). A few participants experienced mild systemic reactions such as fatigue, headaches, myalgia, and arthralgia. No significant laboratory abnormalities were associated with vaccination. No participants developed myopericarditis (clinical or subclinical) despite intensive monitoring with regularly scheduled electrocardiograms, cardiac enzymes, and adverse event solicitation of cardiac symptoms and diary cards. Preliminary immunogenicity cytotoxic T lymphocyte (CTL) activity as measured by the chromium release assay showed both env and gag responses, although the activity against Env was dominant. CTL responses were predominantly CD8+ T cell mediated, with CTL responses being seen in $\geq$ 50% of study participants. While most study participants demonstrated HIV specific reactivity on EIA diagnostic assays, there was sero-reversion at 24 weeks following immunization in 90% of those demonstrating HIV-specific EIA reactivity

e. Future Plans:

Continued cellular and humoral immunogenicity evaluations on collected samples.

4. Title of Research Project: Protocol G, A Cross-Sectional Study to Screen for and Generate Broadly Neutralizing Monoclonal Antibodies from HIV Infected Individuals (RV212, WRAIR #1320)

a. Investigators:

- Professor Punnee Pitisuttithum
  Vaccine Trial Centre
  Faculty of Tropical Medicine
  Mahidol University
  Bangkok, Thailand

- Dr. Somchai Sriplienchan, COL Jerome Kim, LTC Robert Paris, Dr. Mark de Souza and Ms. Patricia Morgan
Department of Retrovirology,
U.S. Component, AFRIMS
Bangkok, Thailand

b. Objectives:

This protocol seeks to generate broadly neutralizing monoclonal antibodies (mAbs) from volunteers who are HIV infected and have broadly cross-reactive serum neutralizing activity. It will also attempt to determine the clinical and laboratory characteristics of HIV infection that correlate with the presence of broadly cross-reactive neutralizing antibodies.

c. Methods:

Volunteers are seen at the AFRIMS Clinical Trials Center in Bangkok. They attend one study visit study every 3 months to receive a clinical assessment, CD4 count, and viral load measurement, as well as collection of PBMC's and plasma for research assays.

This is a cross-sectional, multi-center study with one or two visits. At the first visit blood is drawn for serum to measure neutralizing activity. If there are clinically significant test results, a follow-up visit is scheduled to review all clinically significant test results with the volunteer and plan referrals for further evaluation, care and treatment. Volunteers who have broadly neutralizing antibodies are asked to return approximately 3 months after the screening visit. Blood is drawn for the preparation of PBMCs, clinical testing and to obtain additional serum to confirm that the volunteer continues to have broadly cross-reactive neutralizing antibodies.

d. Results:

The protocol began enrollment in August 2007 and completed enrollment in July 2008. Only a few Thai volunteers were identified with broadly, cross-reactive antibodies. Isolation and production of monoclonal antibodies is ongoing.

e. Future Plans:

AFRIMS is conducting or will conduct genotyping, antibody isolation and purification, and construct a CRF01_AE antibody pool for reagents, in collaboration with IAVI.

5. Title of Research Project: The Molecular Epidemiology of HIV-1 among HIV Blood Testing Clients Attending the Thai Red Cross Anonymous Clinic in Bangkok, Thailand, (RV225, WRAIR #1383)

a. **Investigators:**

- CPT Miguel A. Arroyo
  Department of Retrovirology, U.S. Component, AFRIMS,
  Bangkok, Thailand

- Dr. Sunee Sirivichayakul
  Division of Allergy and Clinical Immunology
  Department of Medicine, Chulalongkorn University,
  Bangkok, Thailand

- Professor Praphan Phanuphak
  Dr. Nittaya Phanuphak
  Thai Red Cross AIDS Research Center (TRCARC),
  Bangkok, Thailand

- Dr. Kiat Ruxrungtham
  Section of Allergy and Immunology, Department of Medicine
  Chulalongkorn University
  Bangkok, Thailand

- Dr. Jintanat Anaworanich
  Department of Retrovirology, U.S. Component, AFRIMS
  and South East Asia Research Collaboration with Hawaii (SEARCH)
  Thai Red Cross AIDS Research Center
  Bangkok, Thailand

- Dr. Francine E. McCutchan and Dr. Paul T. Scott
  Department of Threat Assessment and Epidemiology
  U.S. Military HIV Research Program (USMHRP)
  Rockville, MD, USA

- Dr. Mark de Souza, LTC Robert Paris and COL Jerome Kim
  Department of Retrovirology
  U.S. Component, AFRIMS,
  Bangkok, Thailand

b. **Objectives:**

To describe the molecular epidemiology of HIV among clients undergoing voluntary counseling and testing Thai Red Cross Anonymous Clinic (TRCAC) for using in planning future vaccine trials in Bangkok, Thailand.

c. Methods:

This protocol aims to characterize the molecular epidemic of HIV-1 among clients attending the TRCAC. A total of approximately 3,000 samples and questionnaires were collected by TRCAC between July 1, 2006 and February 28, 2007 from subjects who requested HIV-1 testing and counseling. The HIV test results and questionnaire information will be linked to each sample by a unique number that does not identify the client. HIV-1 genotyping by MHAbce1 will be performed initially in all HIV-1 positive samples. Drug resistance and HIV-1 full-length genotyping may be performed on a subset of specimens of interest to the research team. Statistical analysis will be performed to determine associations between HIV-1 infection, genotype and, behavioral and demographic characteristics among the study population.

d. Results:

The execution of the protocol began on 8th December 2007 in Thailand. Among the 4,405 clients who were tested for HIV, the mean age was 32.2 years (SD 9.4 years) and two-thirds were men. The overall HIV-1 prevalence was 14% and non-CRF01_AE accounted for 29.6% of the infections (4.5%; subtype B, 0.3%; subtype C, 22.2%; CRF01/B recombinant, 2.4%; BC recombinant; and 0.3%; B/C/CRF01 recombinant), but requires verification by sequencing. Factors associated with HIV-1 infection included female gender, MSM risk group, ≥ 25 years of age, current province outside Bangkok, and ≤ high school education level in a multivariate analysis. Those who reported never using condoms during the past 2 years were more likely to have non-CRF01_AE infections (OR: 2.65; 95%CI 0.17-0.86). A ≥ 5% increase in the number of non-CRF01_AE infections was observed in men, MSM, single clients, unemployed clients, those who reported drinking alcohol "every day" and use of addictive substances. These differences were not statistically significant.

e. Future Plans: Verify putative dual infections cases by cloning PCR products of dual-infection cases. Clones will be tested using the MHAbce. Samples that show different clones that react to different subtype specific probes will be sequenced and subjected to phylogenetic analysis.

6. Title of Research Project: HIV Specific Immune Responses in Thai Individuals with HIV Dementia (RV238, WRAIR #1418)

a. Investigators:

- Dr. Victor Valcour
  Department of Internal Medicine, University of Hawaii
  Honolulu, HI, USA

- Dr. Samart Nidhinandana and Dr. Suwicha Chitpatima
  Pramongkutklao Hospital
  Bangkok, Thailand

- Dr. Silvia Kim, Dr. Mark de Souza and Dr. Jerome Kim
  Department of Retrovirology,
  U.S. Component, AFRIMS,
  Bangkok, Thailand

- Dr. Bruce Shiramizu and Dr. Cecilia Shikuma
  University of Hawaii,
  Honolulu, HI, USA

- Dr. Thippawan Chuenchitra
  Division of Research,
  RTA Component, AFRIMS,
  Bangkok, Thailand

b. Objectives:

This is a proposal to obtain pilot data and to assess logistical feasibility for intended Hawaii-Thailand joint NIH Exploratory/Developmental Research proposal on the research topic of HIV-associated dementia (HAD). The proposal is written to include follow-up visits and evaluations to 24 months. There are two main scientific goals: 1) to assess the relationship between monocyte/macrophage activation and dementia in ARV naïve patients living in Thailand and 2) to assess the relationship among proviral DNA levels, blood HIV viral load, CSF HIV viral load, and cognitive status among ARV naïve pateints with and without HIV dementia.

c. Methods:

Thirty volunteers were HIV-1 infected individuals (15 HAD and 15 non-HAD), and 30 matched HIV-negative controls. All individuals underwent neuropsychological testing. Blood draws were obtained and lymphocyte immunotyping, plasma HIV viral load and monocyte phenotyping was performed.

d. Results:

The current study has established that HIV associated Dementia (HAD) is present in patients infected with circulating recombinant form (CRF) 01_AE (subtype E). HAD HAART-naive HIV-1-infected Thais with no active CNS opportunistic infection were screened for cognitive deficits. Fifteen individuals with HAD were identified and were then matched by age, education, and CD4 count with 15 HIV-1-infected non-demented (ND) individuals. All patients then completed the modified WHO international HIV NP battery. An independent review panel confirmed cognitive diagnoses using all available data. Neuropsychological z-scores were calculated using 30 age-, education-, and gender-matched HIV-negative Thais as controls.

Neuropsychological testing abnormalities were identified in most domains among HAD participants compared to HIV-negative controls. Abnormalities were most prominent in tests of verbal learning and recall, visuospatial construction, and selective attention. By contrast, only Color Trails 2 and EIWA Block Design distinguished ND individuals from controls.

The PBMCs from the HAD, non-HAD and HIV-negative controls groups were surface stained with M/M markers CD14/CD16/HLADR and CD14/CD69/HLADR. Non-parametric analysis (Mann-Whitney test) on these subsets of monocyte markers at baseline (V1) revealed significant differences associated with CD14/CD16/HLADR markers between controls and HIV groups (both HAD and non-HAD) ($p < 0.001$). We did not observe differences between HAD and non-HAD groups. We also compared the expression of these markers at V2 (6 months after initiation of ARV) to see if ARV induced could induce any change on expression of these markers of these monocytes. As expected, all individuals responded well to treatment with reduction in both plasmas.

VL and augmentation of CD4+ counts: There was a significant reduction of expression for both percent and absolute number for CD14/CD16/HLADR M/M after ARV treatment in the HAD group and less impressively in the non-HAD group, meeting significance in only measurement of the absolute M/M number.

e. Future Plans:

Follow up for the 15 HAD and 15 non-HAD individuals with continuous collection of data. We are planning to submit a new protocol proposing an expansion of the immunological assessment in a similar cohort to better understand the underlying immunological mechanisms that may cause HAD.

7. Title of Research Project: Predictors of Neuro-Cognitive Decline and Survival in HIV-Infected Subjects (SEARCH 001, WRAIR #1161)

a. Investigators:

- Dr. Samart Nidhinandana (Principal Investigator)
  Department of Internal Medicine
  Section of Neurology, Pharmongkutklao Hospital
  Bangkok, Thailand

- Dr. Victor Valcour (co-Principal Investigator)
  Department of Internal Medicine
  University of Hawaii-Manoa,
  Honolulu, Hawaii, USA

- Dr. Jerome H. Kim, Dr. Silvia Kim, Dr. Mark deSouza and Dr. Jintanat Ananworanich
  Department of Retrovirology

U.S. Component, AFRIMS,
Bangkok, Thailand

b. Objectives:

This protocol seeks to assess predictors of neuropsychological impairment in Thais and assess changes in neuropsychological testing parameters before and after antiretroviral therapy. A sub-study has also been performed to compare cellular responses, particularly activated monocytes, in volunteers with and without dementia.

c. Methods:

This 96-week study enrolled three groups of volunteers: 15 with HIV-associated dementia, 15 with no dementia and 30 HIV-negative controls. Volunteers attend study visits every 6 months to receive a clinical assessment, neuropsychological testing, CD4 count, and viral load measurement, as well as collection of peripheral blood mononuclear cells and plasma for research assays. Statistical analyses are planned at weeks 48 and 96.

d. Results:

All volunteers completed the study follow up in December 2007. Planned analysis at week 48 showed improvement of neuropsychological testing parameters after antiretroviral therapy. Volunteers with HIV-associated dementia performed worst at all time points compared to HIV-infected volunteers without dementia and HIV-negative volunteers. HIV DNA level in peripheral blood mononuclear cells predicted HIV-associated dementia. However, activated peripheral blood monocytes were maintained at high levels in HIV-negative controls and were non-discriminatory for HIV-associated dementia. The baseline data has been published in a peer-review journal and the 48-week data has been accepted as a poster presentation at the Conference on Retroviruses and Opportunistic Infections in Boston in February 2008.

e. Future Plans:

HIV-infected volunteers will be asked to enroll in a follow up protocol at the South East Asia Research Collaboration/The Thai Red Cross AIDS Research Centre. A total of 19 patients have enrolled. They will undergo neuropsychological testing, viral load measurement and archiving of peripheral blood mononuclear cells for HIV DNA level every 6 months for a period of 2 years.

8. Title of Research Project: Preliminary study of early primary HIV infection in high risk cohort (SEARCH 004, HSRRB # A-14273.3)

a. Investigators:

- Dr. Jintanat Ananworanich (Principal Investigator)

Department of Retrovirology
U.S. Component, AFRIMS and SEARCH
Bangkok, Thailand

- Dr. Nittaya Phanuphak (co-Principal Investigator)
  The Thai Red Cross AIDS Research Centre
  Bangkok, Thailand

- Dr. Jerome H. Kim, Dr. Robert Paris, Dr. Mark deSouza and Dr. Miguel Arroyo
  Department of Retrovirology
  U.S. Component, AFRIMS
  Bangkok, Thailand

- Prof. Praphan Phanuphak and Dr. Sunee Sirivichayakul
  The Thai Red Cross AIDS Research Centre
  Bangkok, Thailand

b. Objectives:

This protocol investigated the incidence, demographics, HIV subtype and genotypic resistance in acute HIV infection within a high-risk Thai cohort at the Thai Red Cross Anonymous Clinic (TRCAC), which has an HIV prevalence of about 17%.

c. Methods:

TRCAC uses 4th generation enzyme-linked immunoassay (AxSYM) for HIV diagnosis. This protocol utilized discarded samples from HIV testing that were routinely stored. AxSYM-negative samples were pooled and nucleic acid testing (NAT) was performed using Roche Amplicor v 1.5 ultrasensitive assay. Acute HIV infection samples were AxSYM-negative, NAT positive. In addition, AxSYM positive samples were tested with 1st generation US Food and Drugs Administration approved HIV-1 EIA (HIV-1 Microelisa System, Organon Teknika, Durham, NC). Acute HIV-infection samples were AxSYM-positive, first generation sensitive EIA negative and NAT positive. Demographic and risk behavior data from the TRCAC questionnaires were collected.

d. Results:

Plasma from 6426 clients was tested, and 11 subjects had acute HIV-1 infection. Seven of 5402 AxSYM-negative samples were NAT positive and 4 of 1024 AxSYM-positive samples were 1st generation EIA negative. The acute HIV infection prevalence was 20.3 per 10,000 persons at risk, and the estimated HIV incidence was 2.7 per 100 person-years. Median HIV RNA was 99,601 copies/ml. The majority had CRF01_AE strains and 1 had dual CRF_01AE and B recombinant. No samples showed resistance to antiretrovirals. The majority of the subjects were educated, employed and had income three times higher than the average Thai household monthly income. Few consistently used condoms. This study has completed. The results have been accepted for poster

presentation at the HIV Pathogenesis meeting in Banff, Canada in March 2008. The manuscript has been published in a peer-reviewed journal (J Acquir Immune Defic Syndr. 2008 Oct 1; 49(2):151-5).

e. Future Plans:

A prospective study (SEARCH 010) has been funded by the U.S. Military HIV Research Program to diagnose clients of the TRCAC with acute HIV infection within the first 2-3 weeks of onset of infection. Volunteers with acute HIV infection will be asked to enroll in a cohort study that will follow their clinical, immunological and virological outcomes over a course of 2 years. Volunteers will be offered antiretroviral treatment and the effect of treatment will be assessed. Sub-studies evaluating immunologic and virologic parameters in the central nervous system and genital compartments will also be performed. The study will commence in mid 2008.

9. Title of Research Project: Cohort study of HIV-1 Incidence among clients of the Thai Red Cross AIDS Research Centre, Bangkok, Thailand (RV233, SEARCH 008, WRAIR #1426).

a. Investigators:

- Dr. Nittaya Phanuphak (Principal Investigator)
  The Thai Red Cross AIDS Research Centre
  Bangkok, Thailand
  Department of Retrovirology
  U.S. Component, AFRIMS and SEARCH
  Bangkok, Thailand

- Dr. Jintanat Ananworanich, MD, PhD (co-Principal Investigator)
  Department of Retrovirology
  U.S. Component, AFRIMS and SEARCH
  Bangkok, Thailand

- Dr. Jerome H. Kim, Dr. Robert Paris, Dr. Mark de Souza and Dr. Miguel Arroyo
  Department of Retrovirology
  U.S. Component, AFRIMS
  Bangkok, Thailand

- Prof. Praphan Phanuphak and Dr. Sunee Sirivichayakul
  The Thai Red Cross AIDS Research Centre
  Bangkok, Thailand

b. Objectives:

Assess populations in Thailand at high risk for HIV-1 infection for potential efficacy trials of candidate HIV vaccines to include: (1) assess incidence of HIV-1

infection and volunteer retention; (2) describe early viral load and CD4 counts; (3) assess participant willingness to participate in HIV vaccine efficacy trials and other HIV prevention trials; (4) describe volunteer risk behavior.

Characterize the HIV-1 genotype distribution among HIV-1 seroconverters using MHA technology and selected full-length sequencing, as well as archival of cells and plasma for potential cellular and humoral immune studies in the future.

c. Methods:

Approximately 1200 HIV-negative, male and female clients, ages 18 to 50 years, seeking HIV counseling and testing at the TRCARC will be enrolled to obtain 1000 HIV-negative volunteers for interval assessment of HIV-infection. Participants will receive voluntary counseling and testing (VCT) for HIV-antibody in conjunction with questionnaires on risk behavior, willingness to participate in HIV vaccine trials and other HIV prevention trials at 4 month intervals for a period of 1 year. Weighted incidence estimates will be calculated with exact 95% confidence intervals.

d. Results:

Enrolment began on 1 August 2008 and as of December 31, 2008, 515 subjects had been enrolled with an HIV prevalence of 7%. Follow-up visits had occurred on 41 subjects.

e. Future Plans:

Continue enrollment until target of 1000 HIV seronegative subjects is reached, with follow-up visits.

10. Title of Research Project: Establish and Characterize an Acute HIV Infection Cohort in a Thai High Risk Population (SEARCH 010, WRAIR 1494, RV254)

a. Investigators:

- Dr. Jintanat Ananworanich (Principal Investigator)
  Department of Retrovirology, US Component, AFRIMS and SEARCH
  Bangkok, Thailand

- Dr. Nittaya Phanuphak (co-Principal Investigator)
  The Thai Red Cross AIDS Research Centre
  Bangkok, Thailand

- Dr. Jerome H. Kim, Dr. Robert Paris, Dr. Mark deSouza and Dr. Miguel Arroyo
  Department of Retrovirology
  U.S. Component, AFRIMS
  Bangkok, Thailand

- Prof. Praphan Phanuphak and Dr. Sunee Sirivichayakul
  The Thai Red Cross AIDS Research Centre
  Bangkok, Thailand

b. Objectives:

This protocol investigated the incidence, demographics, HIV subtype and genotypic resistance in acute HIV infection within a high-risk Thai cohort at the Thai Red Cross Anonymous Clinic (TRCAC), which has an HIV prevalence of about 17%.

c. Methods:

TRCAC uses 4th generation enzyme-linked immunoassay (AxSYM) for HIV diagnosis. This protocol utilized discarded samples from HIV testing that were routinely stored. AxSYM-negative samples were pooled and nucleic acid testing (NAT) was performed using Roche Amplicor v 1.5 ultrasensitive assay. Acute HIV infection samples were AxSYM-negative, NAT positive. In addition, AxSYM positive samples were tested with 1st generation US Food and Drugs Administration approved HIV-1 EIA (HIV-1 Microelisa System, Organon Teknika, Durham, NC). Acute HIV-infection samples were AxSYM-positive, first generation sensitive EIA negative and NAT positive. Demographic and risk behavior data from the TRCAC questionnaires were collected.

d. Results:

Plasma from 6426 clients was tested, and 11 subjects had acute HIV-1 infection. Seven of 5402 AxSYM-negative samples were NAT positive and 4 of 1024 AxSYM-positive samples were first generation EIA negative. The acute HIV infection prevalence was 20.3 per 10,000 persons at risk, and the estimated HIV incidence was 2.7 per 100 person-years. Median HIV RNA was 99,601 copies/ml. The majority had CRF01_AE strains and 1 had dual CRF_01AE and B recombinant. No samples showed resistance to antiretrovirals. The majority of the subjects were educated, employed and had income three times higher than the average Thai household monthly income. Few consistently used condoms. This study has completed. The results have been accepted for poster presentation at the HIV Pathogenesis meeting in Banff, Canada in March 2008. The manuscript has been submitted to a peer-reviewed journal.

e. Future Plans:

A prospective study (SEARCH 010) has been funded by the U.S. Military HIV Research Program to diagnose clients of the TRCAC with acute HIV infection within the first 2-3 weeks of onset of infection. Volunteers with acute HIV infection will be asked to enroll in a cohort study that will follow their clinical, immunological and virological outcomes over a course of two years. Volunteers will be offered antiretroviral treatment and the effect of treatment will be assessed. Sub-studies evaluating immunologic and virologic parameters in the central nervous system and genital compartments will also be performed. The study commenced in mid 2008.

11. Title of Research Project: Assessment of Neutralizing Antibody in Participants from phase I/II Trials of ALVAC-HIV (vCP1521) Priming with Chiron gp120 B/E, Sanofi-Pasteur Oligomeric gp160, or AIDSVAX™ B/E gp120 B/E Boosting against a Newly Developed, Standardized Panel of HIV-1 Isolates (RV243, WRAIR #1431)

a. Investigators:

- Chitraporn Karnasuta, PhD and Mark S. de Souza, PhD, MPH
  Department of Retrovirology, US Component, AFRIMS,
  Bangkok, Thailand

b. Objectives:

1. To compare cross-clade NAbs among samples from HIV uninfected volunteers who have received a prime-boost regimen of ALVAC vCP1521 and three different Env subunit protein boosts.

2. To compare the frequency and titers of NAbs induced among the three protein boost regimens.

3. To evaluate the evolution of NAb, i.e. the change in immunogenic responses whether NAb is detectable and/or shows variation in its expression, among volunteers during the prime-boost regimen.

c. Methods:

This study aims to use the TZM-bl pseudovirus neutralizing antibody assay method. Pseudovirus, at 200 $TCID_{50}$, is incubated with various dilutions of test samples for 1 hr at 37°C in 96-well flat-bottom culture plates. Freshly trypsinized TZM-bl cells are added to each well. One set of control wells receives cells plus virus (virus control), and another set receives cells only (background control). After a 48-hour incubation, reconstituted luciferase assay substrate is added to each well for measurements of luminescence using a luminometer. The 50% inhibitory dose (ID_{50}) is defined as either the plasma dilution or sample concentration (in the case of sCD4 and mAbs) that caused a 50% reduction in relative luminescence unit compared to virus control wells after subtraction of background.

d. Results:

The protocol is expected to begin in February 2009. No analysis of data or abstracts has resulted from this study at this time.

e. Future Plans:

Future Plans will depend on study results.

III. APPENDICES:

PERSONNEL ASSIGNED UNDER COOPERATIVE AGREEMENT

Department of Administration
1. Ms. Bung-on Kesdee
2. Mr. Weerasak Yeephu
3. Mr. Sompol Boonnak
4. Ms. Pattrapan Jullasing
5. Ms. Geerati Sornwattana
6. Ms. Yinglak Apisitsaowapa
7. Ms. Somporn Krasaesub
8. Mr. Theerasak Ponepan
9. Mr. Prinya Yoophasook
10. Ms. Khanitha Rojanasthien
11. Ms. Bussara Sukpanichnant
12. Ms. Sasiprapa Pichitphan
13. Mr. Danuphol Junkaew
14. Ms. Usa Panichpathompong
15. Ms. Mattaneeya Pattarateerachai
16. Mr. Eakkachai Trikomol
17. Ms. Panisara Puangsilpa
18. Ms. Tassanee Eamkamon
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13. **Gibbons RV.** *Some not so random thoughts on dengue.* **Emerging Diseases in Southeast Asia. Phramongkutklao Hospital, Thailand. 26 November 2008.**

14. **Gibbons RV.** *Some non-random thoughts on dengue.* **International partners', Forum on Important Viral and Parasitic Infections. College of Public Health, Philippines. 5 November 2008.**

15. **Gibbons RV.** *Field site presentations: epidemiology of incidence of fever, dengue and DENV infections - Kamphang Phet.* **4th Field Site Consortium Meeting, Pediatric Dengue Virus Initiative (PDVI) Field Site Consortium Investigators Meeting. Jeju, Korea. 25-27 September 2008.**

16. **Gibbons RV.** *Social and economic implication of dengue.* **2nd International Conference on Dengue and Dengue Haemorrhagic Fever "Global Innovation to Fight Dengue". Ministry of Public Health, Phuket, Thailand. 15-17 October 2008.**

17. **Harrington LC; Ponlawat A.** *The effect of male age on female lifetime reproductive success of the dengue vector, aedes aegypti.* **American Society of**

Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.

18. **Hirunpetcharat ; Mahakunkijcharoen Y; Jearnwattanalert P; Kittigul L; Mahannop P; Pichyangkul S.** *Effective induction of protective immunity against blood-stage P. yoelii infection by single immunization with a recombinant P. yoelii MSP1(19) formulated with CpG ODN and montanide isa adjuvants in mice.* **Joint International Tropical Medicine Meeting 2008 "Tropical Medicine in the - omics Era". Imperial Queen's Park Hotel, Bangkok, Thailand. 13-14 October 2008:151.**

19. **Islam D; Gettayacamin M; Venkatesan MM; VanGessel Y; Bodhidatta L; Mason CJ.** *Efficacy of WRSd1 Shigella dysenteriae-1 vaccine in a rhesus monkey model of shigellosis.* **Keystone Symposia on Molecular and Cellular Biology 2008, at Royal Orchid Sheraton Hotel, Bangkok, Thailand during 22-27 October 2008. Poster Abstracts no. 153.**

20. **Jarman RG.** *Discussion on BSL-3 AFRIMS experience – planning to operation.* **Biosecurity Engagement Program, BSL-3 Planning, Programming and Operations Symposium. Biosecurity Engagement Program, US Department of State, Sandia National, Laboratories and International Biological Threat Reduction, Manila, Philippines. 14-15 July 2008.**

21. **Jarman RG; Klungthong C; Holmes EC; Chinnawirotpisan P; Rodpradit P; Pimgate C; Scott TW; Rothman AL; Yoon IK; Gibbons RV.** *Genetic analysis of dengue viruses collected from mosquitoes and humans during dengue cluster investigations in Thailand..* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

22. **Jarman RG.** *Genetic diversity of dengue virus at Kampheang Phet, Thailand.* **2nd International Conference on Dengue and Dengue Haemorrhagic Fever "Global Innovation to Fight Dengue". Ministry of Public Health, Phuket, Thailand. 15-17 October 2008.**

23. **Jarman RG; Halstead SB; Thaisomboonsuk B; Gibbons RV; Nisalak A; Rothman AL; Libraty DH; Thomas SJ; Yoon IK; Endy TP.** *Single dilution neutralization assay to determine the dengue sequence of infection impact on disease severity.* **5th Research Network Meeting, Pediatric Dengue Virus Initiative (PDVI). Quebec, Canada. 5-8 June 2008.**

24. **Jeamwattanalert P; Ruang-areerate T; Sunyakumthorn P; Mason CJ; Gaywee J.** *Genetic diversity of Orientia tsutsugamushi from febrile patient in upper regions of Thailand during 2004-2007.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

25. **Kabeya H; Colborn JM; Bai Y; Lerdthusnee K; Richardson JH; Kosoy MY.** *Detection of Bartonella tamiae DNAs in ectoparasites from Thai rodents and their similarity with cultures from human patients from Thailand.* **40th Annual Conference of Vector Ecology. Ft. Collins, Colorado, USA. 28 September - 2 October 2008.**

26. **Kayatani AK; Fukuda MM; Leke RG; Taylor DW.** *Comparison of serological profiles and antibody avidities to 8 major candidate vaccine antigens in Thai and Cameroon adults.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

27. **Klungthong C.** *Advance laboratory diagnosis for dengue infection.* **Epidemiology, Management and Control of Dengue. Department of Tropical Pediatrics, Faculty of Tropical Medicine, Mahidol University, Thailand. 23 July 2008.**

28. **Kosoy MY; Bai Y; Lerdthusnee K; Richardson JH; Boonmar S; Peruski LF; Sutthirattana S; Maloney SA.** *Phylogenetic relations between Bartonella strains identified in animals and humans from Thailand.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

29. **Lerdthusnee K; Leepitakrat S; Insuan S; Monkanna T; Chayapum K; Leepitakrat W; Charoensongsermkit W; Khlaimanee N; Jones JW; Murphy JR; Richardson JH.** *Development of field-deployable chigger surveillance trap.* **XXIII International Congress of Entomology (ICE 2008). Durban, South Africa. 6 -11 July 2008.**

30. **Libraty DH; Capeding RM; Acosta L; Tallo V; Mercado E; Bautista A; Jarman RG; Yoon IK; Gibbons RV; Brion JD.** *A Prospective study of primary dengue virus infections during infancy: preliminary findings.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

31. **May L; Chretien JP; Pavlin J.** *Beyond traditional surveillance: applying syndromic surveillance to developing settings – opportunities and challenges.* **7th Annual International Society for Disease Surveillance (ISDS) Conference. Raleigh, North Carolina. 3-5 December 2008.**

32. **Macareo L; Li Q; Smith B; Polhemus M; Miller S.** *Area under the efficacy curve analysis of intravenous artesunate in treatment of severe malaria.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

33. **Myint KS; Gibbons RV; Kipar A; Jarman RG; Perng GC; Van Gessel Y; Mongkolsirichaikul D; Solomon T.** *Japanese encephalitis virus infection resulted in apoptotic neuronal cell death in a rhesus monkey challenge model.*

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35. **Nakjarung K; Meng CY; Phasuk R; Sethabutr O; Bodhidatta L; Smith BL; Mason CJ.** *Genotype distribution of noroviruses in children with acute gastroenteritis in Cambodia.* **American Society for Microbiology 108th General Meeting. Boston, USA. 1-5 June 2008.**

36. **Nguyen TV; Le KT; Nguyen PM; Phasuk P; Setrabutr O; Vu HB; Le LT; Mason CJ.** *Detection of norovirus and sapovirus in Vietnamese children with diarrhea by real time RT-PCR.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

37. **Nisalak A.** *Overview, laboratory diagnosis of dengue infection.* **Epidemiology, Management and Control of Dengue. Department of Tropical Pediatrics, Faculty of Tropical Medicine, Mahidol University, Thailand. 23 July 2008.**

38. **Nisalak A.** *Serology/virology of dengue.* **WHO Collaborating Center for Case Management of Dengue/DHF/DSS. Queen Sirikit National Institute of Children Health. Bangkok, Thailand. 1 July 2008.**

39. **Nobthai P; Serichantalerg O; Pootong P; Bodhidatta L; Mason CJ.** *Multilocus sequence typing of Campylobacter jejuni isolates from Thailand 1998-2004*. **American Society for Microbiology 108th General Meeting. Boston, USA. 1-5 June 2008.**

40. **Pavlin J.** *Old and new respiratory infections – surveillance by the Armed Forces Research Institute of Medical Sciences (AFRIMS).* **11th Annual Force Health Protection Conference. Albuquerque, New Mexico. 9-15 August 2008.**

41. **Pavlin JA.** *Global health and emerging diseases*. **First Asian and Second National Medical Students' Research Conference, Pune, India. 20-24 February 2008.**

42. **Phasuk P; Nakjarung K; Sethabutr O; Bodhidatta L; Mason CJ.** *An epidemiological study of human caliciviruses (HUCVS) infections, from five different geographical areas of Thailand.* **Joint International Tropical Medicine Meeting 2008 "Tropical Medicine in the -omics Era". Imperial Queen's Park Hotel, Bangkok, Thailand. 13-14 October 2008:162.**

43. **Pilakasiri C; Myint KSA; Supyapoung S; Jarman RG; Gibbons RV.** *Hepatitis antibody profile of Royal Thai Army nursing students.* **18th Asia-Pacific Military Medicine Conference. Singapore. 14-18 April 2008.**

44. **Potts JA; Kalayanarooj S; Nimmannitya S; Srikiatkhachorn S; Nisalak A; Vaughn DW; Endy TP; Libraty DH; Green S; Rothman AL.** *Classification and regression tree (CART) analysis using clinical laboratory variables known to be associated with dengue to establish early disease classification.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

45. **Saeung A; Baimai V; Otsuka Y; Rattanarithikul R; Takaoka H; Choochote W.** *Evidence to support three sibling species of the Anopheles barbirostris form a (Diptera: culicidae) in Thailand.* **Joint International Tropical Medicine Meeting 2008 "Tropical Medicine in the -omics Era". Imperial Queen's Park Hotel, Bangkok, Thailand. 13-14 October 2008:155.**

46. **Scott TW; Carey JR; Fansiri T; Richardson J.** *A residual demography method for estimating age structure of wild mosquito vector populations.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

47. **Serichantalergs O; Pootong P; Bodhidatta L; Dalsgaard A; Pitarangsri C; Guerry P; Tribble DR; Anuras S; Mason CJ.** *Relationship of PFGE, serotype, and multiple antimicrobial resistance among Campylobacter jejuni isolated from travelers and U.S. military personnel in Thailand.* **Joint International Tropical Medicine Meeting 2008 "Tropical Medicine in the -omics Era". Imperial Queen's Park Hotel, Bangkok, Thailand. 13-14 October 2008:162.**

48. **Singhsilarak T; Silapong S; Manik FI; Sethabutr O; Nakjarung K; Yoosuf AA; Mason CJ; Bodhidatta L.** *Detection and genetic characterization of group A rotavirus strains in the Maldives.* **American Society for Microbiology 108th General Meeting. Boston, USA. 1-5 June 2008.**

49. **Smith BL; Polhemus ME; Jongsakul K; Ogutu B; Weina PJ; Miller RS.** *A phase II, randomized, open-label, dose-ranging study of GMP intravenous artesunate for optimizing parasite clearance in uncomplicated P. falciparum malaria.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

50. **Srijan A; Bodhidatta L; Pitarangsi C; Oransathid W; Puripanyakom P; Wongstitwilairoong B; Piyaphong S; Mason C.** *When does enrichment broth need to be used for isolation of Campylobacter species from human diarrhea stool samples?.* **Joint International Tropical Medicine Meeting 2008 "Tropical Medicine in the -omics Era". Imperial Queen's Park Hotel, Bangkok, Thailand. 13-14 October 2008:163.**

51. **Srijan A; Puripunyakom P; Bodhidatta L; Mason CJ.** *Comparison of modified cefsulodin-irgasan-novobiocin agar and macconkey agar for isolation of aeromonas spp. from human stool specimens.* **American Society for Microbiology 108th General Meeting. Boston, USA. 1-5 June 2008.**

52. **Tabprasit S; Thongsen N; Sununtarod E; Seakam K; Sirisopana N; Nitayaphan S; Sangkharomya S; Mason CJ; Pavlin JA.** *Geographic Information Systems (GIS) mapping to track HIV infection rates in Thailand using data from Royal Thai Army recruits 1991-2007.* **2nd Annual Global Partners in Public Health Informatics Meeting "PHI2008--Envisioning Options for Integrated Public Health Information Systems for Low Resource Settings: Components, Connections, Partners, Strategies". Seattle, Washington, USA. 18-19 September 2008.**

53. **Tachibana M; Iriko H; Muratova O; Song G; Wu Y; Sattabongkot J; Takeo S; Otsuki H; Torii M; Tsuboi T.** *Immunization with recombinant proteins of a gametocyte protein Pfs230 expressed using wheat germ cell-free system successfully induce transmission-blocking antibodies against Plasmodium falciparum.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

54. **Takahashi M; Lerdthusnee K; Urakami H; Misumi H; Khlaimanee N; Monkanna T; Chareonsongsermkit W; Leepitakrat S; Kikuchi H.** *Comparative study of the life cycles of the infected and uninfected Leptotrombidium arenicola and L. deliense.* **58th Japan Society of Medical Entomology and Zoology. Osaka, Japan. 17-19 April 2008.**

55. **Tash K; Chaitaveep P; Myers G; Nedek S; Rodpul P; Rangsin R; Bethell D; Smith B; Fukuda M; Meesiri S.** *Outcomes of Smear-Positive Tuberculosis Patients from the Thai-Myanmar Border.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

56. **Thomas S.** *Tetravalent, PDK-derived, live-attenuated vaccine candidates.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

57. **Thomas S; Nisalak A; Libraty D; Sahasakmontri N; Kalayanarooj S; Vaughn D; Putnak R; Endy T.** *Performance of the dengue plaque reduction neutralization test (PRNT) in primary and secondary dengue virus (DENV) infections across a spectrum of test conditions.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

58. **Thomas SJ.** *Measuring cell-mediated immunity: meeting report.* **Dengue Vaccine Scientific Forum, World Health Organization. Belem, Brazil. 23-24 April 2008.**

59. **Thomas SJ; Lynch J; Innis B; Kenneth E.** *Status of the WRAIR/GSK live attenuated dengue vaccine.* **Joint International Tropical Medicine Meeting 2008 "Tropical Medicine in the -omics Era". Imperial Queen's Park Hotel, Bangkok, Thailand. 13-14 October 2008.**

60. **Thomas SJ; Nisalak A; Libraty DH; Sahasakmontri N; Kalayanarooj S; Vaughn DW; Putnak R; Endy TP.** *Performance of the dengue plaque reduction neutralization test in primary and secondary dengue virus infection.* **5th Research Network Meeting, Pediatric Dengue Virus Initiative (PDVI). Quebec, Canada. 5-8 June 2008.**

61. **Thomas SJ; Lynch J; Innis B; Eckels K.** *WRAIR/GSK bio tetravalent live virus dengue vaccine.* **2nd International Conference on Dengue and Dengue Haemorrhagic Fever "Global Innovation to Fight Dengue". Ministry of Public Health, Phuket, Thailand. 15-17 October 2008.**

62. **Thomas SJ.** *Dengue vaccine development: 2008 update.* **30th Annual Congress of the Malaysian Paediatric Association. Malaka, Malaysia. 21-24 August 2008.**

63. **Ubalee R; Jin X; Asher C; Carroll D; Jenwithisuk R; Sattabongkot J; Richardson J; Kozar MP; Melendez V.** *Metabolism of CYP450 markers of enzymatic activity and primaquine by the induced HC-04 immortalized hepatic cell line.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

64. **Velasco JM.** *Influenza surveillance in Cebu.* **National Forum on Influenza Pandemic Preparedness and Response. Research Institute for Tropical Medicine, Department of Health, Philippines. 4 June 2008.**

65. **Vithessonthi K; Pancharoen C; Thisyakorn C; Nisalak A; Thisyakorn U.** *Seroepidemiology of dengue virus infection in HIV-infected children in comparison to healthy children.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

66. **Wongstitwilairoong B; Srijan A; Oransathid W; Bodhidatta L; Mason CJ.** *A Comparision study of the efficiency of plating media and enrichment broths for the recovery of salmonella species from human stool samples.* **American Society for Microbiology 108th General Meeting. Boston, USA. 1-5 June 2008.**

67. **Wongstitwilairoong T; Gaywee J; Sirisophana N; Mason CJ; Pavlin JA.** *Mining pattern model of influenza surveillance.* **7th Annual International Society for Disease Surveillance (ISDS) Conference. Raleigh, North Carolina. 3-5 December 2008.**

68. **Wongstitwilairoong T; Pavlin JA; Gaywee J; Siripana N; Mason CJ.** *Influenza pattern model by using data mining technique.* **32th The Association of Medical**

Technologist of Thailand Conference 2008 (AMTT 2008) . Ambassador City Jomtien Hotel, Thailand. 6-9 May 2008:161.

69. **Woodring JV; Srijan A; Mason C.** *Prevalence of vibrio, aeromonas and salmonella species in various seafoods from supermarkets and fresh markets in Bangkok, Thailand.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

70. **Youngpakoo K; Thaisomboonsuk B; Jarman RG; Jittmittraphap A; Jampangern W.** *Binding characterizations of Southeast Asian dengue 2 virus and American dengue 2 viruses with single E gene amino acid substitutions.* **Joint International Tropical Medicine Meeting 2008 "Tropical Medicine in the -omics Era". Imperial Queen's Park Hotel, Bangkok, Thailand. 13-14 October 2008:160.**

71. **Yoon IK.** *Dengue epidemiology in Kamphaeng Phet*, **Thailand. Joint International Tropical Medicine Meeting 2008 "Tropical Medicine in the -omics Era". Imperial Queen's Park Hotel, Bangkok, Thailand. 13-14 October 2008.**

72. **Zhu G; Gao Q; Zhou H; Li J; Lu F; Liu Y; Jetsumon J.** *Susceptibility of Plasmodium vivax to Anopheles anthropophagus from different areas in China.* **American Society of Tropical Medicine and Hygiene 57th ASTMH Annual Meeting. New Orleans, LA, USA. 7-11 December 2008.**

LECTURE 2008

1. **Myint KSA**. *Ecology and epidemiology of viral hepatitis.* **Lecture. Master of Public Health Students, Faculty of Public Health, Mahidol University. Bangkok, Thailand. 31 October 2008.**

2. **Nisalak A.** *Arboviruses and dengue virus.* **Lecture. The Second Year Medical Cadets, Phramongkutklao College of Medicine. Bangkok, Thailand. 19 August 2008.**